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BY

R. W. MARSH

AND

I. THOMAS

WITH THE ASSISTANCE OF

F. C. BAWDEN J. T. MARTIN

M. J. R. HEALY E. A. PARKIN

D. J. WATSON

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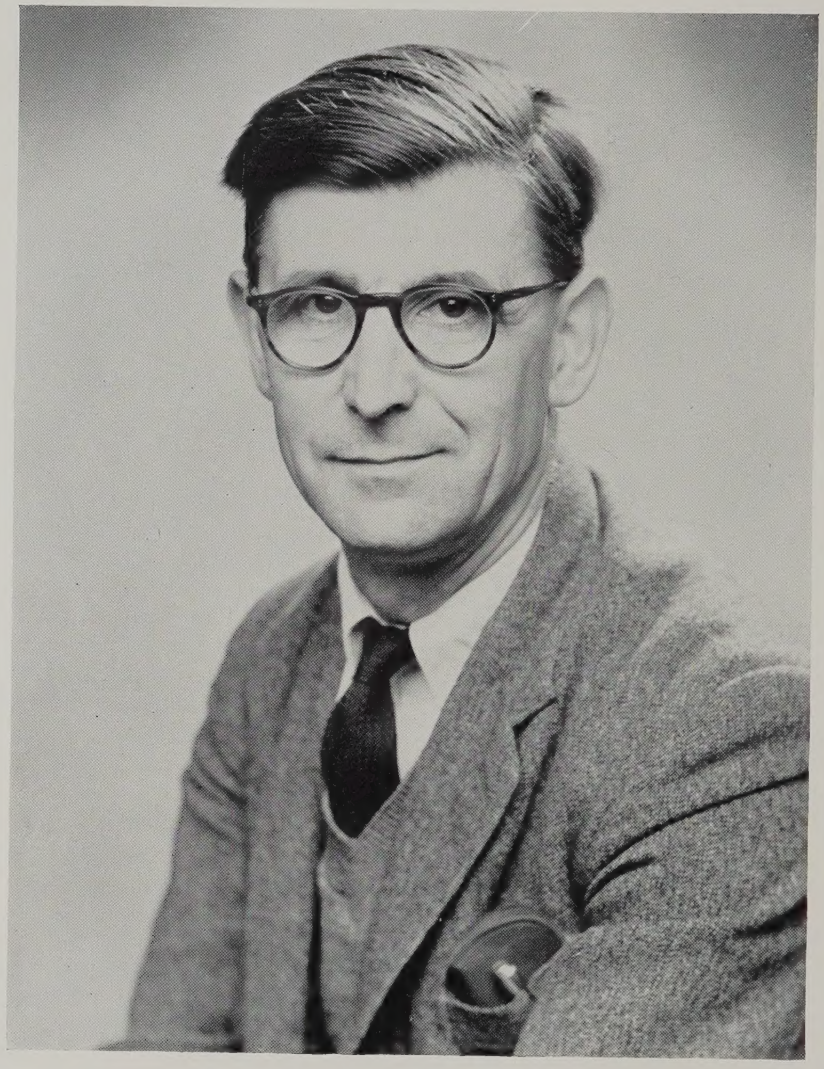

C. POTTER

President of the Association of Applied Biologists, 1960–61

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The future of chemical control of insects

By C. POTTER

Address of the President of the Association of Applied Biologists delivered to the Annual General Meeting on Friday, 14 April 1961

This talk is primarily on the chemical control of insects. I am only considering other methods of control when they are affected by chemical control, or might be used together with it; but I want to make it clear that I think these non-chemical methods have also a valuable part to play in their own right.

Speculation is a hazardous procedure and any reasoned forecast can only be based on the existing situation and the events which led up to it. I therefore propose to review the major developments in control of insects to date and the problems which have arisen and to try to discern the present trends. Then it should be possible to speculate whether progress will continue in the same direction and what new problems are likely to be encountered in this direction, or whether new ways of using chemicals for the control of insects may emerge to deal with current problems.

Before the discovery of the high insecticidal activity of DDT and BHC just about 20 years ago, neither commercial firms nor research workers generally, in the field of insecticide research, had much faith in the large-scale development of cheap synthetic chemicals for insect control. Derivatives of arsenic were used as persistent stomach poisons; petroleum oils, tar oils and the plant derivatives, pyrethrum, nicotine and the derris group were used as contact poisons. Hydrogen cyanide, ethylene oxide, carbon disulphide and ethylene dichloride, trichloro-ethylene and carbon tetrachloride were commonly used fumigants. Tattersfield and his colleagues (Gimingham & Tattersfield, 1927; Tattersfield, 1928) at Rothamsted had pointed out the possibilities of 2,4-dinitro-*o*-cresol for killing insect eggs and of naphthalene for killing insects in the soil and some attempt was being made to develop the thiocyanates as contact insecticides (Potter & Musgrave, 1940).

With the range of chemicals and techniques then available, control of many species of insect pests was neither profitable nor possible and the control obtained of many others was partial and inefficient.

The problem of the chemical control of the pests of stored foods and other stored products was perhaps the easiest, because the material to be protected is concentrated into a small space, because fumigation could readily be used and potent fumigants were available. But even here, many species were virtually unchecked. The techniques and science of fumigation were being developed by Page and his colleagues (Page & Lubatti, 1939), but pyrethrum was the only contact insecticide that appeared useful and for which an effective formulation and technique of application had been worked out (Potter, 1935).

Crops were largely unprotected from insect pests, except for some of relatively high value, such as top and soft fruit and glasshouse and market garden crops. Even

within this restricted range, protection was often very inefficient. There was almost no chemical control practicable for agricultural crops apart from the use of derris for control of flea beetle on brassicas.

Partial control could be obtained of some of the pests associated with man and domestic animals, but it was not possible even to consider large-scale chemical control of such insects as the deadly-disease-carrying mosquitoes and tsetse flies.

With the introduction of the synthetic chlorinated hydrocarbons DDT and BHC and the organophosphorus insecticides TEPP, parathion and schradan, the extent to which insecticides were used increased very rapidly.

For the first time, relatively cheap, highly potent contact insecticides were available in quantities which could be rapidly expanded to meet any demand. Insecticides derived from plants, such as derris and pyrethrum, are relatively inflexible in this respect.

Not only did the new synthetic chemicals extend the range of species susceptible to chemical control, but their physical and chemical properties were such that two techniques of using insecticidal chemicals, previously of limited application, because of lack of materials with suitable chemical and physical properties, became of widespread practical importance and greatly increased the possibilities of highly effective economic control.

The first technique was the formation of a persistent residual film of insecticide which killed insects coming into contact with it. This technique was not new. Pyrethrum had been used as a residual film to control moth pests in warehouses and the possibilities of the method pointed out (Potter, 1938). But pyrethrum is unstable to light and air and the possibilities remained limited until the advent of the persistent chlorinated hydrocarbons. Stable non-volatile insecticides such as DDT could form a residual film effective for periods ranging from several days to several months, even when exposed to the full effects of weathering. The technique of using residual protective films was useful in all the major fields of applied entomology: medical, household, veterinary, stored products and plant protection.

The second technique was restricted to plant protection. It was to use chemicals that are absorbed into and translocated by the plant, the systemic insecticides. This technique enabled insects which could not be dealt with effectively in any other way, to be reached and killed by the chemical. It was also possible to make a whole plant lethal to a given species of insect over a period, even when it was growing rapidly. Here again, the idea of systemic action was not new and selenium compounds had been shown to act in this way and kill aphids and red spider mites (Hurd-Karrer & Poos, 1936). But the high toxicity of selenium to man, together with its persistence in soils and plants, made it very dangerous and greatly limited its use. Currently used organophosphorus systemic insecticides are also toxic to man but they are broken down to non-toxic substances and, with due precautions, it is possible to use them safely on a wide range of crop plants. The technique of systemic action is now being extended to the control of veterinary pests.

With the new chemicals and the scope of some techniques greatly enlarged, it appeared that the way was clear to control all the major insect pests and there was a vast expansion in the use of chemicals for insect control.

A great deal of success has been achieved and the dangers, difficulties and problems

should not blind us to it. In a recent publication of the W.H.O. expert committee on malaria (Anon., 1954*b*) it was stated that of a total world population of 2800 million, 1300 million live in malarious or formerly malarious regions. Malaria has been eradicated from areas inhabited by 280 million people, while a population of over 697 million is now covered by malaria eradication programmes. Preliminary programmes are being started for a further 138 million. While insecticides are not the sole means of malaria eradication they form an important part of it. The newer insecticides have also been important in greatly reducing the incidence of typhus and preventing epidemics, particularly in time of war, by the control of lice. The use of DDT for louse control in combination with vaccination reduced typhus incidence in Guatemala and Mexico from nearly 3000 cases per annum in 1945 to 8 in 1951 (Anon., 1954*a*). The new chemicals have also made easier the control of many other species of public health, veterinary, household and stored products importance.

In the field of plant protection it is difficult to overestimate their importance in safeguarding and increasing our food supply. Catastrophic losses due to insect attack are now a rarity and crops once hazardous to grow because of insect attack can now safely be grown.

Reliable figures to show how much the new insecticides have increased crop yields and have made it possible to increase the world food supply are difficult, if not impossible, to obtain. However, data are available which leave little doubt that the use of the new synthetic insecticides has resulted, at least in certain instances, in greatly increased yields. Even more important perhaps, they have made it possible, either directly or indirectly, to produce satisfactory crops in areas where this could not be done before (Noone, 1958). It has been pointed out that, in the United States, the curve for increased production per acre became much steeper for a number of agricultural products following the introduction of the new synthetic insecticides which occurred on a large scale about 1945 (Noone, 1958). It has also been stated (Tharp, 1957) that in the United States 15,000,000 acres can now produce the same quantity of cotton that 40,000,000 acres produced during the First World War. Part of this improvement is undoubtedly due to other causes, but the new insecticides play a considerable part. In South Carolina the new chemicals for wireworm control raised the yield of wheat from 0–10 bushels to the acre to 25–50 bushels/acre. Data from America also show that crops can now be grown in situations where it has not previously been possible; it has been stated that over 80 high-vitamin crops at present being grown in the United States could not be grown without the use of the new chemicals to control their pests (Noone, 1958).

Indirectly crop production has been increased, because the new chemicals have made areas safe for man to cultivate, where previously he had to risk his life if he entered the area. The World Health Organization, reporting in 1954 the first Asian Malaria Conference of 1953 (Anon., 1954*b*) stated that for many years there was a slogan in Afghanistan 'If you want to die go to Qundus'. The slogan was justified, for the farmers that tried to work the rich lands of the Qundus were inevitable victims of severe if not fatal attacks of malaria. Land sold in 1935 for 4 Afghanis/acre (about one shilling and sixpence). In 1952 the price was 5000–10,000 Afghanis/acre (about £80–159 sterling), an increase due largely to malaria control.

I have dwelt so far on the benefits that have resulted from developments in chemicals and techniques, but the path that once seemed straight and clear has been found full of obstacles and dangers.

At first it was thought that with the new weapons at our disposal, pest species could be totally eradicated and then the insecticide could cease to be used. This has not so far proved possible as a general rule. In special cases the insecticide has been used to reduce a population and then the environment has been radically altered by, for example, sanitation and general hygiene, so that it has become unsuitable for the pest. Then permanent eradication can occur. However, for plant pests in general and other pest species also, this is not usually possible.

Where chemicals alone are relied on for control or attempted eradication, one major obstacle has been the occurrence of resistant strains. Again the phenomenon is not new; scale insects resistant to HCN and codling moth resistant to arsenic were serious problems before the introduction of the newer synthetic compounds (Brown, 1961). But it had not been realized how adaptable an insect is. It now seems possible that any insect species can develop resistance to any chemical, although it is not easy to predict the speed at which it will develop or the degree of resistance that can occur. As a result of the development of resistance there are now a number of instances where a chemical at first gave efficient and economic control, but later had to be abandoned because it was no longer economically effective. The problem of resistance has occurred in all the major fields of applied entomology (Brown, 1961).

Resistance has been shown most dramatically in the field of public health. At least fifty arthropod species of public health importance have developed resistance to the chlorinated hydrocarbon or organophosphorus insecticides. The problem is said to be most serious in the anopheline vectors of malaria (Brown, 1960*a*) of which sixteen species have developed resistance to dieldrin and four to DDT and dieldrin. Resistance is of importance, not only in the housefly (*Musca domestica* L.) which has become resistant to a number of insecticides in many parts of the world, but also in the body louse *Pediculus pediculus humanus* L. vector of typhus, human flea (*Pulex irritans* L.) and two spp. of bedbugs (*Cimex lectularius* L. and *C. hemipterus* Fabr.).

Resistance is a serious problem in the veterinary field, particularly if, for the purposes of the argument, ticks may be included (Brown, 1961). At present there are no serious resistance problems in the control of stored products insects, but there are indications that some might develop (Parkin, 1959).

There are now many instances (sixty-five including mites) of plant pests becoming resistant and here also, particularly if the mites may be included, the development of resistance has presented serious problems in the control of pests of a number of crops. Recent records include DDT resistance in the Codling moth, small cabbage white butterfly, potato flea beetles, Colorado potato beetle and diamond back moth, TDE resistance on the red banded leaf roller, aldrin and dieldrin and heptachlor resistance in the onion fly and parathion resistance in the walnut, green peach and spotted alfalfa aphids (Brown, 1961). However, in spite of the use of insecticides, many plant pests have not developed resistance and I believe that in England, although some resistance has occurred with red spider mites, there is no confirmed example of resistance with insect pests of outdoor crops. If the life cycle is long and only a proportion

of the total population in the area is exposed to the poison, which is not applied frequently, and if the treated insects are interbreeding with untreated insects, the problem of resistance may not easily arise.

A second problem, greatly aggravated by the vastly increased scale of use of insecticides, has been the risk of toxicity to man. A number of the earlier insecticides were highly toxic to man and presented hazards both during application and to the consumer. For instance, legislation was necessary to control the residues of arsenic on apples. However, there are now very many highly toxic chemicals and they are used on a very wide range of edible crops; the risks are therefore much more widespread. I have been told, but I have not verified the story, that in a survey carried out in the United States some years ago, residues of DDT were found in every restaurant meal sampled and DDT was found in the body fat of every person tested. There is therefore a need for constant vigilance to ensure that the chemicals for insect control are used in such a way as to afford the minimum risk to human health.

The risk is not confined to human beings but also occurs with domestic animals and wildlife, and this could be said to be a third major problem in the use of insecticides.

Where insecticides are applied by 'blanket' spraying over large areas, such as treating forests for defoliating caterpillars or in large-scale attempts at eradication such as the campaign against the imported fire ant *Solenopsis saevissinia* in the south-east United States, damage to wildlife is very liable to occur unless highly specific chemicals can be used. Evidence of damage to wildlife has been provided during control or eradication programmes involving mass applications of insecticide on spruce budworm, gypsy moth, Mediterranean fruit fly and grasshoppers and mormon crickets (Cope & Springer, 1958). Damage does occur also with more limited applications and there has been a great deal of publicity in this country about birds being poisoned through eating cereal seeds dressed with insecticide.

The lethal effects of insecticides on the more obvious forms of wildlife is certainly a problem that must be looked into and ways and means of safeguarding birds and mammals must be actively sought, but it would not seem wise to take any severe restrictive action until sufficient reliable data have been accumulated to assess the extent of the damage and weigh it against the benefits conferred.

It is also highly desirable to examine the possibility that the widespread use of pesticides is having a marked effect on less obvious fauna of the field, the forest, the hedgerow, pond and streams. The Nature Conservancy has for some time been interested to foster a study of the effects of plant-protective chemicals on wildlife and now has formed a team to work on this subject.

The fourth problem is to prevent the insecticide from destroying beneficial insects. Considerable destruction of pollinating insects such as the honey-bee can occur and it is not easy to assess the economic loss produced in this way. In the course of some work on the problem of bees being poisoned in the field, we acquired some evidence of the widespread occurrence of the modern persistent insecticide. We were looking for bees which were free from insecticide to act as controls in bioassay work and obtained evidence that all our own bees contained traces of a persistent insecticide. We then obtained samples from a number of different areas and all were shown to contain traces of this insecticide. These bees all appeared to be perfectly normal.

However, probably the most important effects are produced by the destruction of the parasites and predators of the pests. There have been many instances where the destruction of predators and parasites by the use of a chemical to control a particular pest has resulted in another species, previously a minor pest, becoming a major one. A rather less obvious result of the destruction of parasites and predators is that if chemical control of a particular pest is started and it destroys the parasites and predators of the pest as well as the pest itself, failure or discontinuance of the chemical control may lead to serious outbreaks of the pest, in the interval between removal of the chemical check and the building up of the biological check.

The present position is therefore that, compared with the position before the war, chemicals are far more effective and can be profitably and extensively used in all the major fields of pest control. But the greatly increased use of these chemicals has intensified and extended the problems associated with their use. The major problems are the formation of strains of insects resistant to insecticides, the toxicity to humans and wildlife, the destruction of natural checks on the populations of pests and potential pests, and danger to pollinating insects.

If future developments of chemicals applied to kill insects are considered, it seems likely that progress will be made in finding chemicals and techniques of using them which will be at least as effective as those in current use against the pest species, but which will reduce or overcome the four major problems just outlined.

Considering first the problem of toxicity to human beings, the increased seriousness of the problem has arisen, not so much from the higher toxicity to man of the modern synthetic insecticides (lead arsenate, nicotine and HCN are as dangerous as these) but from the very widespread use of these chemicals.

I think this problem will be reduced if not entirely solved by the introduction of chemicals with a greater selectivity between insects and man. They will be highly toxic to insects and selectively non-toxic to man. This trend is already apparent in the organophosphorus insecticides where compounds much safer than the originally introduced parathion and TEPP are now available. A contributory safety factor, which is likely to be developed, is to find chemicals which are sufficiently persistent to achieve the insecticidal effects but then decompose to harmless products.

The problem of mammalian toxicity alone would appear to give sufficient justification for intensive study of the biochemical interactions of insecticides both with insects and with mammals. By this means alone can the gap between toxicity to man and toxicity to the insect be widened on a rational basis. At present the process must be largely empirical, which is wasteful of time and effort and only certain of results in the long term.

The problems of toxicity to beneficial insects and to wildlife have many aspects in common and may be considered together. Probably for a long time to come we will not have a range of selective products, each of which is toxic to a given pest species and not toxic to beneficial insects or any form of wildlife. The bacteria or viruses or their toxins that attack insects could provide some selective controls for some species if they can be successfully and economically used in practice, but will probably have only a limited range of importance. Even if a range of highly selective chemicals were available they might be uneconomic to produce.

Chemical selectivity will undoubtedly play a part in reducing the problem, but it seems likely that physical selectivity and improved application techniques will prove more important. By physical selectivity is meant any physical property of the chemical itself, or a formulation of it, that can be used to confer selectivity.

As with mammalian toxicity, physical selectivity can be achieved by using chemicals which are just persistent enough to kill the pest species and then decompose to harmless products. This approach can only be used when the pest is in an attacking stage for a very short period and that period can be accurately estimated.

Selectivity of another kind may be achieved by application techniques. A number of systemic insecticides are toxic by direct contact as well as via the plant tissue. Such insecticides applied as foliage sprays may kill many more insect species than if applied locally in the soil to be taken up by the roots. It is also clear that broadcast applications of insecticides to control soil pests may have much more far-reaching side effects than localized treatments in the form of seed dressings.

In the future, serious toxic effects on beneficial insects and wildlife might be largely avoided by choosing insecticides with the right amount of persistence, preparing a specific formulation, and carefully timing their application and localizing the treatment where it will produce the maximum effect on the pest species with the minimum side effects. Selectivity obtained by choosing chemicals with the appropriate physical properties and formulating them and applying them to produce a selective effect may be collectively referred to as ecological selectivity.

The problem of insect resistance seems to be the most intractable. Where there is a big reduction in the pest population produced by the insecticide and the survivors can only mate with one another, resistance to a greater or lesser degree appears to develop, irrespective of the chemical used. The degree of resistance produced and the speed with which it is produced seems to depend on the chemical and the population of insects.

Some techniques of application would appear liable to produce resistant strains more readily than others. Where persistent insecticides are used as residual films, the situation can arise that no susceptible insect in a given population can survive and the resistant survivors, of necessity, mate with one another. If the persistent insecticide is not selective, natural biotic checks will also be removed.

Because an insect that has become resistant to one chemical may also have become resistant to a group of other chemicals—cross-resistance (Brown, 1961)—or become resistant to other chemicals very quickly, the problem may become more and more difficult as time goes on.

So far the chief method of dealing with the problem has been to wait until resistance develops and then look for another effective chemical. However, workers in the United States are considering the possibilities of using two or more insecticides, preferably chemically unrelated, in a rotation to control a given pest species, in the hope that this will either delay or prevent the appearance of resistant strains (Glass, 1960).

Some other possibilities for dealing with this problem have been suggested. One is to use insecticides where resistance is negatively correlated, that is to say the individuals resistant to one chemical are susceptible individuals to another chemical and vice versa (Ascher, 1958; Brown, 1960*b*). Some of the most convincing evidence that this might be possible has been provided by Japanese work (Ogita, 1961). It has been found that

phenylthiourea (PTU) is more toxic to DDT-resistant fruit flies (*Drosophila*) than to a normal strain. It has also been shown that the greater susceptibility to PTU originates at the gene locus 65 on chromosome 2, the same locus that confers greater resistance, not only to DDT but also to BHC, chlordane, parathion and some other insecticides. Further it was shown that selection pressure with PTU causes the resistant strain to revert to susceptibility. Unfortunately PTU does not have this effect with house-flies and is not a sufficiently good insecticide for practical use (Brown, 1960*a*, *b*). Insecticides with negatively correlated resistance could be used together or alternately. Another possibility is to use physical agents such as desiccating dusts, either alone or in combination with chemicals. Presumably chemically resistant individuals are likely to be as susceptible to the physical agents as normal individuals. It is too early to judge the practical value of these possibilities.

Another idea for preventing resistance developing, which applies chiefly to the field of plant protection, is to try to use chemical and biological control in such a way that they are complementary. It is assumed that eradication is not possible and that the objective is to keep the pest population below the level at which it will cause economic damage. The idea is to find chemicals and techniques of application which will keep the pest population in check, but will not greatly affect the parasites and predators. The insecticide is applied so that it will not reduce the pest population below the level where it will attract the parasites and predators and maintain them in sufficient numbers to form a check. The parasites and predators will prevent any large increase in pest population and will not discriminate in favour of chemically resistant individuals.

The idea has been put forward that predators and parasites might be selected for resistance and therefore continue to function in spite of the application of the insecticide. In Canada the attempt has been made to select in the laboratory DDT-resistant *Macrocentrus ancylivorus* Roh., a useful parasite of oriental fruit moth (Pielou & Glasser, 1951). So far as I know this line of thought has not yet led to any results of practical value but it has not been very much explored.

The idea of using chemicals in such a way that the biotic checks on all the pest species in the ecosystem continue to function, is a desirable aim, whether resistance problems are concerned or not (Hagen & Smith, 1958). A programme where the use of a systemic insecticide has been successfully combined with biotic checks, has been worked out for the control of spotted alfalfa aphid in California. In most of California native predators, introduced predators and entomogenous fungi now keep the aphid below the economic threshold for most of the year and the systemic insecticide, applied in such a way as to be selective, is only used when the population rises above the economic threshold.

A combination of chemical control and the release of sterilized males might also help to prevent the occurrence of resistance. Work on eradication of the screw worm *Callitroga hominovorax* (Cqrl.) has shown that the technique of releasing sterile males can be very effective at low population densities and could therefore be a useful non-chemically selective procedure to follow chemical treatment (Knipling, 1955; Cornwell & Bull, 1960).

Before finally trying to estimate future general trends in the use of chemicals to

control insects, it seems worth while to consider the use of chemicals other than direct killing agents. Chemicals which affect the fertility of the insect have been found and are being further investigated (Knipling, 1960). It seems possible that chemicals that affect other vital functions such as finding and feeding on the host plant or feeding mechanisms in general, might prove useful and might be more readily made selective than the lethal agents. One reason for the current lack of such chemicals is that special screening tests would have to be devised to look for them.

While, judging from past experience, repellent chemicals would appear to be useful only as an aid to insect control in special circumstances, there might be a considerable future for attractants (Green, Beroza & Hall, 1957). Attractants are used as a research tool and there is probably room for their development as an auxiliary in chemical control by enabling the presence of a few insects to be detected and, by this early detection of the presence of the pest, to help the timing of control measures. Their possible usefulness for attracting the pest to a trap or poison and destroying it in this way has long been recognized, but not greatly explored, perhaps again because techniques of assessing the possible usefulness of materials used in this way are laborious and difficult. Recently there has been a renewed interest in this technique which can be highly selective. Where sex lures are used, attractants may assist if there is likely to be a resistance problem. As long as there is an effective method of destroying the insect once it has been attracted, it seems unlikely that any selection of individuals that fail to respond to a sex lure would present a serious problem, since they would not be likely to mate anyway.

Attractants have been used recently with success in a campaign for the eradication of Mediterranean fruit fly *Ceratitis capitata* (Wied.) in Florida (Green *et al.* 1957).

An interesting possibility of the use of attractants is for the control of tsetse flies. Experiments have been made by treating cattle with insecticide and using them as perambulating poison baits (Hocking, 1960). If successful, this technique would provide a good example of selectivity and effectiveness produced by a combination of application method and attractant and lethal chemicals, based on a knowledge of the behaviour of the pest species. This could be contrasted with the non-selective method of 'blanket' spraying or fogging with the lethal chemical alone.

To sum up, it appears that, leaving aside the ever-present need for greater effectiveness in use and cheapness and ease of application, the need for the future is to find new ways and means of controlling insects which offer less risks of toxicity to man, beneficial organisms and to wildlife. In addition the attempt must be made to find chemicals and ways of using them which will delay the appearance of resistant strains, or prevent their occurrence.

Probably the present procedures adopted by those searching for new chemicals for pest control will result in an increasing number of compounds with a high toxicity to insects and a low toxicity to man. It will be necessary to have such chemicals available with a wide range of chemical and physical properties so that they may cover the range of requirements throughout the field of pest control. The basic need in chemical control of insects now appears to be the need for greater selectivity. There are two ways of doing this, first, by means of selective chemicals, secondly, by more selective usage. In the field of stored products entomology the selectivity primarily required is between

man and insect, apart from this, general insecticidal action is required; but in the other fields of entomology, particularly plant protection, a much greater degree of selectivity is required. If it is assumed that insect-control chemicals of low toxicity to man will be found, these chemicals must be selective between the pest and other organisms when applied. As I have previously pointed out, insecticides can be selective because they are toxic to one species and not to another; this has been called physiological selectivity. But a chemical that is not physiologically selective may be made selective by the way in which it is used, and I have used the term ecologically selective to cover this form of selectivity although this term has previously been used as a somewhat narrower definition. Ecological selectivity can be obtained by using a chemical with the right degree of persistence, by taking advantage of systemic action, by local placement or formulation so that the chemical is only available to the pest species, and using attractants in combination with the lethal chemical.

Where a high degree of selectivity has been obtained, either by means of physiological selectivity or ecological selectivity or a combination of the two, problems such as toxicity to wildlife should be greatly reduced and because the natural biological checks should remain in operation, the outbreaks at present caused by the removal, either of the checks of the pest being controlled, or of other potential pest species present in the ecosystem, should not occur. For the same reason the problem of resistance should be reduced because biological control would remain in operation.

I think that the commercial firms which have served us so well in the past will continue to play their part in providing us with highly effective lethal insecticides with a range of chemical and physical properties and with greater physiological selectivity, at least between man and insect. But I think that there is need for much more research to obtain greater ecological selectivity and to find out what are the potentialities for chemicals, other than lethal agents.

Almost by definition, this means more detailed studies of the biology of the pest species, of the ecology of the environment in which it lives and the factors influencing its numbers. Detailed knowledge of its physiology, particularly behavioural physiology, is also necessary. I think that while much of this work should be done as a general study, more should be done with the specific object of finding out what sort of chemicals may be useful and how and when they should be used to obtain the maximum effect in preventing damage to the pest with the minimum deleterious side effects. But this is only the essential background. If chemicals other than lethal agents seem likely to prove useful, their properties and function must be clearly defined and techniques for finding them and assessing their usefulness worked out, particularly to enable commercial firms to search for such chemicals. Formulation, application technique and timing can all play an important part in ecological selectivity as well as in the economical and effective use of any pest-control chemical, and there is room for much research on these subjects with the objective of increasing selectivity and effectiveness.

It is on trying to find out the sort of chemicals that are useful and how best to use them, alone or in combination, that I think the emphasis should be in the future, rather than on the search for more and more lethal agents. Much of this work is long-term and must rely on government support if it is to be done.

I believe that the use of chemicals for insect control has resulted in a very great

alleviation of human misery and saved very many lives; it has also made our food supply much more secure and made possible greatly increased food production. The benefits conferred have greatly outweighed the drawbacks and disadvantages. At times I think the people who violently criticize the present use of insecticides might temper their criticism if they reflected on just how much misery, pain, death and starvation has been prevented by means of them.

I hope we may learn to overcome at least some of the drawbacks and disadvantages, so that chemicals for the control of insects can be used with even greater success in the future.

REFERENCES

Anon. (1954*a*). Typhus control in Guatemala and Mexico. *Chron. World Hlth Org.* **8**, 23.

Anon. (1954*b*). Report of the first Asian Malaria Conference. *Chron. World Hlth Org.* **8**, 117.

Anon. (1961). Eighth Report of the expert Committee on malaria. *World Hlth Org. Techn. Rep. Ser.* no. 205, 3.

Ascher, K. R. S. (1958). A common information source for all resistance problems in biology is urgently needed in view of the paucity of effective counter-measures to insecticide resistance. *Indian J. Malar.* **12**, 615.

Brown, A. W. A. (1960*a*). The resistance problem, vector control and W.H.O. *Misc. Publ. ent. Soc. Amer.* **2**, 59.

Brown, A. W. A. (1960*b*). Mechanisms of resistance against insecticides. *Ann. Rev. Ent.* **5**, 301.

Brown, A. W. A. (1961). The challenge of insecticide resistance. *Bull. ent. Soc. Amer.* **7**, 6.

Cope, O. B. & Springer, P. F. (1958). Mass control of insects. The effects on fish and wildlife. *Bull. ent. Soc. Amer.* **4**, 52.

Cornwell, P. B. & Bull, J. O. (1960). Insect control by gamma-irradiation: an appraisal of the potentialities and problems involved. *J. Sci. Fd Agric.* **11**, 754.

Gimingham, C. T. & Tattersfield, F. (1927). Laboratory and field experiments on the use of 3:5-dinitro-*o*-cresol and the sodium salt for winter spraying. *J. agric. Sci.* **17**, 162.

Glass, E. H. (1960). Current status of pesticide resistance in insects and mites attacking deciduous orchard crops. *Misc. Publ. ent. Soc. Amer.* **2**, 17.

Green, N., Beroza, M. & Hall, S. A. (1957). Recent developments in chemical attractants for insects. *Advances in pest control research*, III, 129. New York and London.

Hagen, K. S. & Smith, R. F. (1958). Chemical and biological methods of pest control. *Agric. Chem.* **13** (7), 30, 89.

Hocking, K. S. (1960). The control of tsetse flies with insecticides. *Rep. seventh Commonw. ent. Conf., London*, p. 128.

Hurd-Karrer, A. M. & Poos, F. W. (1936). Toxicity of selenium-containing plants to aphids. *Science*, **84**, 252.

Knipling, E. F. (1955). Possibilities of insect control or eradication through the use of sexually sterile males. *J. econ. Ent.* **48**, 459.

Knipling, E. F. (1960). Views on future research contributing to a solution of the resistance problem. *Misc. Publ. ent. Soc. Amer.* **2**, 157.

Noone, J. A. (1958). Pesticides' impact on food production and consumption. *J. agric. Fd Chem.* **6**, 735.

Ogita, Z. (1961). Relation between the structure of compounds and negatively correlated activity. (Genetical and biochemical studies on negatively correlated cross-resistance in *Drosophila melanogaster*. II.) *Botyu-Kagaku*, **26**, 18.

Page, A. B. P. & Lubatti, O. F. (1939). Recent developments in fumigation of buildings, stored foods and other materials. *Chem. Ind.*, **17**, 1001.

Parkin, E. A. (1959). Insects and stored food—World losses and control measures surveyed. *Food Manufacture*, April 1959, p. 1.

Pielou, D. P. & Glasser, R. F. (1951). Selection for DDT tolerance in a beneficial parasite, *Macrocentrus ancylivorus* Roh. I. Some survival characteristics and the DDT resistance of the original laboratory stock. *Canad. J. Zool.* **29**, 90.

POTTER, C. (1935). An account of the constitution and use of an atomised white oil—pyrethrum fluid—to control *Plodia interpunctella* Hb. and *Ephestia elutella* Hb. in warehouses. *Ann. appl. Biol.* **22**, 769.

POTTER, C. (1938). The use of protective films of insecticide in the control of indoor insects, with special reference to *Plodia interpunctella* Hb. and *Ephestia elutella* Hb. *Ann. appl. Biol.* **25**, 836.

POTTER, C. & MUSGRAVE, A. J. (1940). Some preliminary experiments with β-butoxy-β′-thiocyanodiethylether as an industrial insecticide. *Ann. appl. Biol.* **27**, 110.

TATTERSFIELD, F. (1928). The decomposition of naphthalene in the soil and the effect upon its insecticidal action. *Ann. appl. Biol.* **15**, 57.

THARP, W. H. (1957). Cotton-growing in the U.S.A. *World Crops*, **9**, 139.

Ann. appl. Biol. (1961), **49**, 403–411
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The disfiguration of painted surfaces by fungi, with special reference to *Phoma violacea*

By D. E. EVELEIGH*
Department of Botany, University of Exeter

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SUMMARY

In a survey of moulds attacking painted surfaces, *Phoma violacea* was found commonly associated with paintwork in glasshouses (twenty-eight isolates) and was once observed on exterior paintwork. No source of infection was traced, except for the attack on exterior paintwork which probably originated from a nearby glasshouse. Distribution by insects was noted. *P. violacea* was shown capable of attacking paint films from either surface (above or below) and penetration of the film from the top surface was demonstrated in laboratory and natural exposure tests.

P. violacea was used in standard methods of testing fungicidal paints in comparison with other genera commonly employed. The development of strains resistant to fungicides was noted.

INTRODUCTION

Moulds causing disfiguration of painted surfaces have been troublesome to paint manufacturers for the last 50 years. On outside paintwork *Pullularia pullulans* (de Bary) Berkh. is the principal agent. Klens & Lang (1956) showed that of over 2000 mildewed paint chippings examined, 95 % had *P. pullulans* as the major cause of 'paint spotting'. Similar results have been reported by Reynolds (1950), Vicklund & Manowitz (1951) and Richardson & Olgilvy (1955). Interior paintwork supports a more varied flora with *P. pullulans* again as the principal colonizer, but other genera occur, more especially *Cladosporium herbarum* Link and *Phoma* spp. Undoubtedly, disfiguration is occasionally due to the growth of yet other genera, e.g. *Fusarium* spp., *Helminthosporium* spp. and *Cephalosporium* spp., but many saprophytic species reported have probably merely been associated with detritus on the paint film. Most work in this field is directed towards control measures, more specifically towards control of *Pullularia pullulans*. The present investigation is concerned with the lesser known paint-attacking fungus *Phoma violacea* (Bertel) Eveleigh, syn. *P. pigmentivora* Massee.

Many isolated reports of the occurrence of *P. violacea* have been made (Massee, 1911 and Grove, 1913—England; Haensler, 1921—U.S.A.; Wurth, 1940—Germany), and Nicot-Toulouse (1953) suggests that it is probably a widespread saprophyte. In this investigation twenty-eight isolates were obtained, and several reports were received from widespread localities in Britain. Its presence has been noted in virtually every

* Present address: National Research Council of Canada, Halifax, Nova Scotia, Canada.

glasshouse visited by the author since the investigation began and has been seen several times on interior paintwork of window frames of dwelling houses both in Britain and Canada.

EXPERIMENTAL

Isolates of *P. violacea* were obtained from disfigured paint chippings taken in general from interior situations (Eveleigh, 1961) where it was frequently accompanied by *Pullularia pullulans* and *Cladosporium herbarum* (Table 1). *Penicillium* spp. were frequently isolated but were considered as non-specific moulds growing on organic debris associated with paint films. However, instances of profuse growth of *Penicillium* spp. on emulsion paints in opened containers were noted. The paint chippings were examined under vertical illumination, discoloration due to mould growth and other factors (e.g. dirt particles) thus being readily distinguishable (Goll, Snyder & Birnbaum, 1952). The macroscopic characters of the main fungal genera are fairly distinctive, *Pullularia pullulans* forming a somewhat spotted coating consisting of black chlamydospores singly or in clusters (Pl. 1, fig. 1): *Cladosporium herbarum* appears similar in dry situations but more generally is found in a thick-walled mycelial state. *Phoma violacea* on oil paints is unmistakable because it induces a characteristic red–violet stain, while pycnidia are often found erupting through the paint film (Pl. 1, fig. 2). Most isolations of *P. violacea* were made from white lead paint on wood, but isolates were also obtained from paint covering metal surfaces.

Table 1. *Fungi isolated from disfigured paints*

Species	Interior situations (84 sites including 30 glasshouses)	External situations (3 sites)	Emulsion paint (opened containers)
Cladosporium herbarum Link	53	3	—
Pullularia pullulans (de Bary) Berkh.	31	3	—
Phoma violacea (Bertel) Eveleigh	28	1	—
Phoma spp.	9	—	—
Alternaria tenuis Nees	3	—	—
Alternaria spp.	—	1	—
Penicillium notatum Westling	—	—	1
P. nigricans series	—	—	1
Penicillium spp.	—	—	3
Cephalosporium sp.	—	—	1

Occasional isolates of general saprophytes were made, probably associated with organic debris. Genera included *Cephalosporium*, *Fusarium*, *Penicillium*, *Stemphylium* and *Trichoderma*.

Source of infection

Saprophytic species of *Phoma* are of worldwide distribution, having been found in soil (Gilman, 1957), textiles (Zuck & Diehl, 1946), air and even in butter (Grimes, O'Connor & Cummins, 1932). The source of *P. violacea* could therefore be in widely differing substrates and those associated with natural infections were tested. The paints and linseed oil should theoretically be free from infection, as linseed oil is maintained at high temperatures during processing. Plating of several batches of white lead paint

and of different types of linseed oil on corn-meal agar did not reveal any *Phoma* spp. *P. herbarum* has been found in a glasshouse water main (Bewley & Buddin, 1921) and the water in glasshouses showing natural infection of *P. violacea* was therefore tested by filtration through sterilized cotton-wool pads in wire holders fitted to the taps, fungal spores being trapped on the pad. The pads were then shaken with sterile water, and the latter dilution plated on corn-meal agar, but no isolates of *Phoma* spp. were ever obtained. Dilution plates made from soil and compost samples from these same glasshouses failed to reveal isolates of *P. violacea*. Air exposures using corn-meal and malt agar plates were carried out periodically throughout the investigation at Exeter and daily air exposures were also carried out in Glasgow and Birmingham for a week (October 1958). No pigmented *Phoma* isolates were obtained.

The gelatinous nature of aggregated *Phoma* spp. spores renders them ill-adapted for distribution by air currents, though Richards (1956) reports an air spora in Britain containing an average of 1·1 % of spores of Sphaeropsidales. Haensler (1921) reported that when dead gnats were placed on white lead paint and exposed in laboratory tests, infection of the paints by *P. violacea* was severe. He also noted with regard to natural infection, 'a very luxuriant growth of *P. pigmentivora* has been observed which seemed to be limited very sharply to areas containing insect remains'. In the present investigation it was observed that dead insects in glasshouses were often centres of origin of *P. violacea* infections but these infections were not limited to the peripheral zone around the insect bodies. Certain genera of the Sphaeropsidales have been reported as parasites of insects (Fitzgerald, 1943; Steinhaus, 1946, p. 395). In the present investigation, examination of flies, beetles and weevils collected from glasshouses revealed no trace of parasitism, but plating of a variety of insect remains on corn-meal agar yielded cultures of *P. violacea*. There is still the possibility that a symbiotic relationship between fungus and insect, such as has been suggested by Steinhaus (1955), may exist between scale insects and a 'form' of *P. pullulans*. At least passive distribution by insects appears to be a factor in the dispersal of *P. violacea*.

Although no isolates of *P. violacea* were obtained from soil, water or compost taken from infected glasshouses there is no reason why such locations should not act as continuing reservoirs of infection. The mycelium penetrates deeply into the wooden window frames, and remains as a centre of infection later attacking freshly applied paint. *P. violacea* has been reported in the glasshouses of a Devon smallholding for 40 years in which painting was carried out annually (Knight, 1957). Glasshouses must therefore be regarded as potential infection sources of this fungus. The source of infection of the solitary instance noted on an external wall was traced to a nearby glasshouse.

Mode of attack of paint films

The attack of paint films is governed by several factors besides those generally stated as optimal for growth of fungi. Age affects both hardness of the film (Shapiro, 1958) and the physical state of the drying oil. This latter point is of particular significance initially, as deposition of debris and spores are greatest during the tacky state. Micro-climate at the paint surface is important, primary infection occurring in cracks and crevices, where spores and organic detritus lodge easily and high humidity tends

to persist. For example, primary infection by *P. violacea* was observed on an exterior painted wall originating in holes left by the scaffolding supports.

The micro-climate can be affected by the absorptive nature of the film, oil paint films being capable of absorbing up to one and a half times their own weight of water (Browne, 1953). Thus both humidity at, and hardness of the film are affected by this factor. Laboratory and field experiments were carried out to determine the mode of infection of *P. violacea*, bearing such factors in mind.

Laboratory experiments

Two groups of experiments were carried out using a white lead paint incorporating alkali-refined linseed oil and relatively susceptible to fungal attack.

Group A. Sterile plaster of Paris pats (2 in. diam. × $\frac{1}{4}$ in. thick) were moistened with a Czapek-Dox salts solution and inoculated individually with spore suspensions of *P. violacea* (strains 3 and 7) and *Aspergillus niger*. The pats were then incubated at 25° C. in Petri dishes containing a little sterile water. One series of pats (four per fungus) was painted after 18 hr. incubation and the second series painted after 7 days. When dry, the painted pats were incubated at 25° C. for 8 weeks in sterile Petri dishes with a few ml. of sterile water. A similar complete series, using soft-wood blocks (four per fungus) was also arranged. When this method is used, the oil paint placed directly on a colony of *Phoma violacea* will develop a characteristic red–violet stain within a few hours, owing to the solution of the pigment in the oil of the paint. Growth must be assessed not on general discoloration, but on presence of mycelium within the film. For comparison, a series of wooden blocks was painted and when dry, inoculated with strains of *P. violacea* on the paint surface. These were incubated in Petri dishes as described above.

Group B. Sterile glass slides and wooden blocks were painted with varying numbers of coats (1–6) of white lead paint. A nutrient (1 % malt extract solution) was added to the substrate before painting (one-third of the replicates), or to the paint surface after painting (one-third of the replicates), whilst no extra nutrient source was added to the remaining third. Spore or mycelial suspensions of *P. violacea* strains 1, 2, 3 and 7, were placed individually on the slides and blocks at the following times: (*a*) before painting (1–6 coats); (*b*) after two coats of paint had dried, with two further coats applied over the spore inoculum; (*c*) on the dried paint surface (1–6 coats); (*d*) on the wet (unpolymerized) paint surface. Four replicates of each treatment were made, the treated slides and blocks being kept in sterile glass containers and maintained at 100 % humidity by periodical spraying with sterile water.

The results from group (A) tests showed *P. violacea* to be extremely destructive to paint films put over active fungal colonies. The 18-hr.-old colonies failed to penetrate the film except in one instance, while the week-old colonies penetrated the film and formed pycnidia on the surface. However, it must be noted that these results were obtained with only 50 % of the replicates, the remainder failing to show any signs of attack. The control painted wood blocks, inoculated on top of the dried paint surface, showed pycnidium formation occurring deep in the wood, i.e. penetration from outside the film had occurred. In group (B) tests, no infection of the paint occurred on any of the replicates after 6 months' exposure.

Tropical chamber experiments

Two series of experiments were carried out: in (i), sterilized filter papers (Whatman No. 1) and softwood blocks were painted with white lead paint, while in (ii) sterile filter papers were inoculated individually with spores of *P. violacea* strains and then painted over. Two filter papers were inoculated with strain 7 between two coats of paint. The painted papers were suspended in the tropical chamber, being interleaved with plain pieces of paper, while the blocks were placed obliquely against a galvanized wire support on a low bench. The samples were handled as little as possible and always at the edges, and examined at monthly intervals. The tropical chamber was arranged in this instance to give a cyclic action of a 6 hr. hot period (29° C. and a relative humidity of 92%), and an 18 hr. 'cool' period (24–25° C. and 98–100% R.H.). The micro-flora of the tropical chamber included species of *Aspergillus*, *Penicillium*, *Botryodiplodia*, *Graphium*, *Paecilomyces*, *Fusarium* and *Chaetomium*. *Phoma violacea* was present as a natural infection and also a number of unidentified bacteria.

(i) *Uninoculated treatments.* None of the filter-paper samples showed attack by *P. violacea* after 9 months' exposure. Two of the painted wooden blocks showed incipient attack by *P. violacea* after 14 weeks' exposure. They were then removed from the chamber and kept at 25° C. in a moist container where they showed further progressive attack. Pycnidia at first appeared on the surface of the paint and later developed in the wood, thus again demonstrating penetration of the film from above. Of the remaining seven wooden blocks, two showed attack by *P. violacea* after 8 months' exposure, while the remaining five remained free of such attack up to the end of the experiment (9 months). General infection of the filter papers by *Pullularia pullulans* and *Cladosporium herbarum* occurred after 8 weeks exposure but never became severe. Paint samples infected with *Pullularia pullulans* and *Cladosporium herbarum* were wax-embedded and sectioned but neither mould was observed to have penetrated beneath the film.

(ii) *Paper inoculated beneath the paint. Phoma violacea* strain 2 grew through the paint film after 5 weeks' exposure, mycelial tufts appearing on the surfaces. None of the remaining seven isolates penetrated the paint film by the end of the experiment (7 months).

Natural-infection experiments

Sterilized softwood blocks were painted with white lead, zinc oxide (three types) and titanium dioxide paints using alkali-refined linseed oil as a vehicle. The painted wooden blocks were exposed by attaching them to wooden battens, which were then fitted between glasshouse window frames, one surface of each block being in rough contact with the glass. Exposures were carried out for 5 months in the glasshouses of the Botany Department, University of Exeter (10–32° C.) and at a commercial cucumber grower's hot-houses (15–28° C.) where *P. violacea*, *P. pullulans* and *Cladosporium herbarum* were present on the paintwork. Only slight infection was present in the University glasshouses where linseed oil which was used as a covering to the teak structure supported profuse growths of *C. herbarum*. Scrapings taken from the wooden window frames failed to show any *Phoma* spp.

After 5 months' exposure, the titanium dioxide and white lead paints had been attacked at both sites by *Pullularia pullulans*, *Cladosporium herbarum* and *Phoma violacea*. Slight infection of the zinc oxide paints was present, including attack by *P. violacea*, the stains produced by the latter being mainly blue. Blocks in one glasshouse, in which the humidity was low, did not show any signs of fungal attack. Sections of the disfigured paint films were taken (10–15 μ), the only mould showing penetration of the film being *P. violacea* (Pl. 1, fig. 3).

Resistance to fungicides

The effectiveness of paints against fungal attack can be demonstrated by accelerated laboratory tests and by long-term natural exposure tests. The present-day necessity of screening large numbers of fungicides has led to the general use of the speedier laboratory tests. One of the basic methods employed uses filter papers that have been double coated on both surfaces with paint, cut into 1 in. squares, sterilized in 95% alcohol and placed on a mineral salts (Czapek-Dox) or malt agar. Both agar and squares (two per plate) are inoculated by spraying with a standard fungal suspension and then incubated at 25–30° C. for 3–6 weeks. Variations of this method have been described depending on the type of inoculum used, e.g. *Pullularia pullulans* (Nuodex, 1952); *Aspergillus niger* and *A. oryzae* (Vicklund & Manowitz, 1947); *A. niger*, *A. terreus*, *A. oryzae* and *Trichoderma* sp. (Richardson & Del Giudice, 1952). A clear zone of inhibition around the test piece is not necessarily indicative of an effective paint fungicide and often merely characterizes the latter's moderate solubility in water and high leaching rate. Results are therefore based on fungal growth (percentage cover) on the painted filter-paper squares, though when using white lead oil paints some difficulty is experienced in obtaining 100% coverage of control test pieces.

This method was adopted to compare the effectiveness of standard paint fungicides against several common paint fungi including *Phoma violacea*, pure inocula of *Aspergillus niger*, *Cladosporium herbarum*, and *Pullularia pullulans* and also mixed inocula of *Penicillium notatum*, *P. terrestre* and *P. chrysogenum*. Mixed inocula using *Aspergillus* spp. or *Penicillium* spp. with *Phoma violacea* were not used, as antagonism between them was noted.

The following fungicides incorporated into oil paints were used at the recommended concentrations of 1·0 and 2·0%: (*a*) di-(phenyl mercury)dodeceny! succinate (DPMDS-Super Ad-It (Nuodex)); (*b*) 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine (DCCAT-Nuozene); (*c*) p-toluene sulphonamide (reported by Leonard & Pitman (1951) as highly fungicidal in varnish); (*d*) dehydroacetic acid (reported by Pfohl (1956) to show high fungicidal activity in bread wrappers); (*e*) phenyl mercury acetate (PMA). Some proprietary brands of fungicidal paints were also used in comparison, though the composition and concentration of fungicide in them was not disclosed.

The results showed that 1% of DPMDS, DCCAT, PMA and the proprietary products gave reasonable protection to the paints. Mixed and pure fungal cultures gave very similar results, though slight variation was noted between strains in pure culture (Pl. 1, fig. 4, Table 2). *P. violacea* strains showed no greater resistance than *Pullularia pullulans* strains, while the *Cladosporium herbarum* strains showed less resistance than

these other two genera. Dehydroacetic acid and *p*-toluene sulphonamide were ineffective as fungicides in oil paints, fungal growth occurring at the 2% level. The results with the latter fungicide support those obtained by Arnold & Clarke (1956) using emulsion paints. Long-term incubation brought about the development of fungicide-resistant strains on all paints tested (see Johnson, 1955). Resistant strains of *C. herbarum*, *Phoma violacea* and *Pullularia pullulans* were noted on paints containing 1 % DCCAT, 0·75 % DPMDS, 1 % PMA and also on the proprietary fungicidal paints tested (Pl. 1, fig. 5). During the laboratory tests carried out, occasional *Penicillium* spp. were found growing on the test paints including those containing fungicides. Resistant strains were obtained of *P. brevicompactum* Dierkx., *P. citrinum*

Table 2. *Evaluation of fungicides—laboratory test method*

(Incubation of 4 weeks at 25° C.)

Species	Control white lead paint	Fungicides: DPMDS 0·5 %	DPMDS 1·0 %	DCCAT 0·5 %	DCCAT 1·0 %	PMA 1·0 %
Aspergillus niger	2	C	C	1	C (+)	C
Penicillium spp.	2	1	C (1)	1	1	C
Phoma violacea No. 3	1	C	C	0	0 (3)	C
P. violacea No. 52	2	2	2	1	1	—
Pullularia pullulans No. 56	3	1	C	1	1	C
P. pullulans No. 60	2	0	C	1	—	C
Cladosporium herbarum No. 3	0	C	C	C	C	0
C. herbarum No. 73	0	C	C (r)	0	0	C

Fungicides: DPMDS—di-(phenyl mercury)dodecenyl succinate. DCCAT—2,4,-dichloro-6-(*o*-chloroanilino)-*s*-triazine. PMA—phenyl mercury acetate. Rating (% coverage): 4–100 to 75 %, 3–74 to 50 %, 2–49 to 25 %, 1–25 to 1 %, denotes edges invaded at one or two places, 0—no coverage. Figures in brackets indicate occasional observations, C—inhibition zone around test piece, 'r' indicating development of resistant colonies in such zones, '+' paint edges occasionally attacked.

Thom, *P. spinulosum* Thom and also *Aspergillus versicolor* (Vuill.) Tiraboschi, respectively, from white lead paint containing 1 % DCCAT, 1 % PMA, 0·75 % DPMDS and 1 % DCCAT. Natural exposure tests were carried out in glasshouses using similar paints to those used in laboratory tests. Exposure of the painted wooden blocks was carried out for 5 months. Infection of the blocks occurred but was never severe on the main surface, although the ends of the blocks (i.e. across the wood grain), usually supported fair growth. Black streaks across the main paint surface originated from these areas at the top of the blocks (Pl. 1, fig. 6). Cultures of *C. herbarum*, *Phoma violacea*, and *Pullularia pullulans* were isolated from these streaks, the last-named predominating. A bacterium was also consistently isolated and could be demonstrated adhering to the fungal hyphae.

DISCUSSION

Fungal infection of paint films can originate either from above or below the paint film. In either case, primary infection appears to be dependent on the presence of nutrients from external sources, since it is virtually impossible to induce attack of paint films on inert substrates (Galloway, 1955). The negative results obtained with *Phoma violacea* using painted glass slides support this idea, though isolates were

obtained in this investigation from white lead paint covering a metal surface, and Grove (1913) reports seeing it on bath enamel. Natural infection is rapid when paint is applied directly over an active fungal colony, but *P. violacea* has also been shown here to be capable of penetrating the film from above the surface, and subsequently developing on the substrate beneath. Rupture of the paint film is later caused by mechanical pressure, pycnidia erupting through the film. Rupture of the paint film by mechanical pressure has also been demonstrated by Drescher (1958) using *P. pullulans* and has been noted by the author on a paint specimen at the C.M.I., Kew by *Botryodiplodia theobromae*. Since *Phoma violacea* is often found as a deep-seated infection of wooden window frames, effective control is difficult. Even if the mould can be killed by application of fungicides, oil paints cannot be applied directly, as the red–violet pigment will still diffuse from the dead mycelium and rapidly discolour the paint.

The ability of *Pullularia pullulans* to grow on exterior paints is thought to be partially due to its resistance to both desiccation and ultra-violet radiation. Evans & Bobalek (1956) have demonstrated that in cultures of *P. pullulans* infected with *Penicillium* spp., the latter can be eliminated by exposure to ultra-violet radiation on several successive days. Similar resistant properties of angiocarpic fungi on exposed cotton duck fabric have been reported by Zuck & Diehl (1946) and presumably apply also to the *Phoma* spp. occurring on paint films.

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REFERENCES

ARNOLD, M. H. M. & CLARKE, H. J. (1956). The evaluation of some fungistats for paint. *J.O.C.C.A.* **39**, 900.

BEWLEY, W. F. & BUDDIN, W. (1921). On the fungus flora of glass-house water supplies in relation to plant diseases. *Ann. appl. Biol.* **8**, 10.

BROWNE, F. L. (1953). The absorption of water, swelling and solubility of free films of paint. *J. Forest Prod. Res. Soc.* **3**, 108.

DRESCHER, R. F. (1958). Micro-biology of paint films. IV. Isolation and identification of the microflora on exterior emulsion paints. *Amer. Paint. J.* **42**, 80.

EVANS, R. M. & BOBALEK, E. G. (1956). Accelerated tests for mildew resistance of oil paints. *Industr. Engng Chem.* (*Industr.*), **48**, 122.

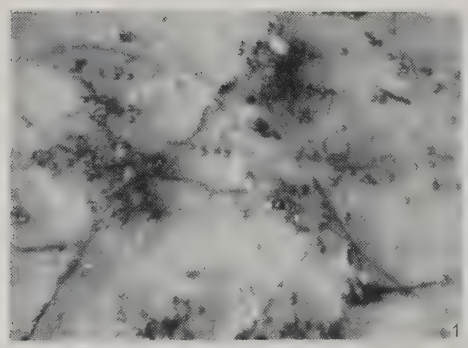
EVELEIGH, D. E. (1961). *Trans. Brit. mycol. Soc.* (In the Press.)

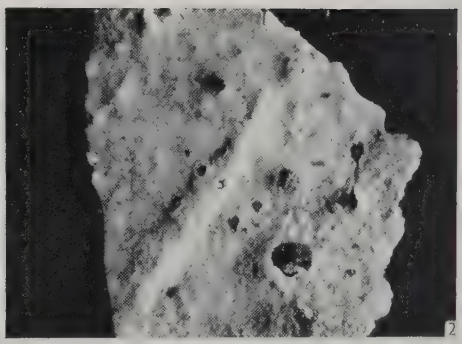
FITZGERALD, L. R. (1943). *Phoma stenobothri*, a fungus parasite of the grasshopper. *Amer. Midl. Nat.* **29**, 761. (*Rev. appl. Mycol.* **23**, 106, 1944.)

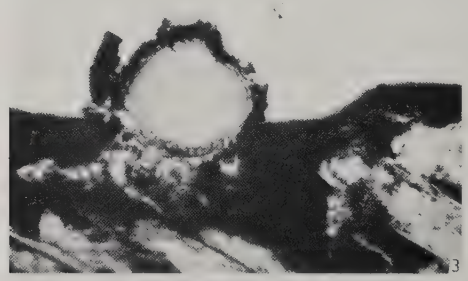
GALLOWAY, L. D. (1955). The growth of moulds on painted surfaces. *J.O.C.C.A.* **38**, 250.

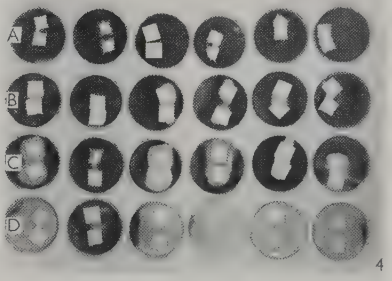
GILMAN, J. C. (1957). *A manual of soil fungi*, 2nd ed. Iowa State College Press.

GOLL, M., SNYDER, H. D. & BIRNBAUM, H. A. (1952). New developments in the diagnosis of paint mildew. *Off. Dig.* no. 326, 149.





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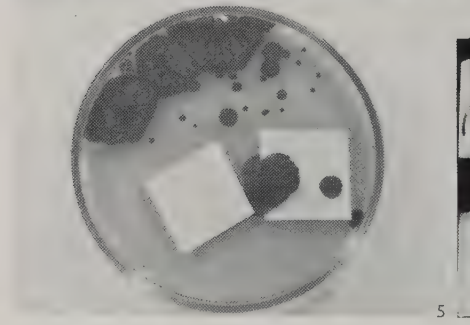

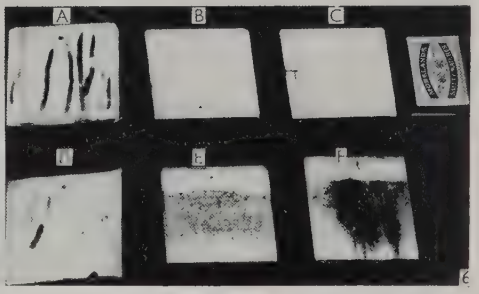
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GRIMES, M., O'CONNOR, M. & CUMMINS, H. A. (1932). A study of some *Phoma* species. *Trans. Brit. mycol. Soc.* **17**, 97.
GROVE, W. B. (1913). Mycological Notes. *J. Bot., Lond.*, **51**, 43.
HAENSLER, C. M. (1921). *Fungus injury to paint surfaces* (Thesis). Rutgers College and the State University, New Jersey.
JOHNSON, F. D. (1955). Are mildew strains fighting back? *Canad. Paint Varn. Mag.* **29**, April, pp. 18, 57.
KLENS, P. F. & LANG, J. F. (1956). Microbiological factors in paint preservation. *J.O.C.C.A.* **38**, 887.
KNIGHT, W. (1957). Personal communication.
LEONARD, J. M. & PITMAN, A. L. (1951). Tropical performance of fungicidal coatings—a statistical analysis. *Industr. Engng Chem.* (*Industr.*), **43**, 2338.
MASSEE, G. (1911). A new paint-destroying fungus—*Phoma pigmentivora*. *Kew Bull.* **8**, 325.
NICOT-TOULOUSE, J. (1953). Deux *Phoma* isolés sur plâtre humide. *Bull. Soc. mycol. Fr.* **68**, 352.
NUODEX PRODUCTS CO. INC. (1952). Pamphlet. *Paint mildew and its control.*
PFOHL, R. R. Chairman, Chicago Club Technical Committee. (1956). Deterioration of protective coatings due to molds and bacteria. (Bibliographic study.) *Off. Dig.* **28**, 954.
REYNOLDS, E. S. (1950). *Pullularia* as a cause of deterioration of plastic surfaces in S. Florida. *Mycologia*, **42**, 432.
RICHARDS, M. (1956). A census of mould spores in the air over Britain in 1952. *Trans. Brit. mycol. Soc.* **39**, 431.
RICHARDSON, J. H. & DEL GIUDICE, V. J. (1952). The testing and use of fungicided paints in food processing plants. *Mod. Sanit.* **4**, March, p. 32, April, p. 32.
RICHARDSON, J. H. & OLGILVY, W. S. (1955). Antimicrobial penetrant sealers. *Appl. Microbiol.* **3**, 277.
SHAPIRO, S. (1958). The evaluation of fungicides for use in paints. *Off. Dig.* **30**, 414.
STEINHAUS, E. A. (1946). *Insect microbiology*, p. 395. New York: Comstock Publ. Co.
STEINHAUS, E. A. (1955). Observations on the symbiotes of certain coccidae. *Hilgardia*, **24**, 185.
VICKLUND, R. E. & MANOWITZ, M. (1947). Test methods for evaluating paint fungicides. *Amer. Paint. J.* **32**, 67, 70, 72, 75, 78, 80, 82, 86.
VICKLUND, R. E. & MANOWITZ, M. (1951). Mildewing of paint and its prevention. *Paint Varn. Prod. Mgr*, **31**, 8 and 26.
WURTH, K. (1940). Violet spots on white paints. *Farbenztg.* **45**, 518.
ZUCK, R. K. & DIEHL, W. W. (1946). On fungal damage to sun-exposed cotton duck. *Amer. J. Bot.* **33**, 374.
J.O.C.C.A.—Journal of the Oil and Colour Chemists' Association.
Off. Dig.—Official Digest of the Federation of Paint and Varnish Production Clubs.

EXPLANATION OF PLATE

Fig. 1. *Pullularia pullulans* growing on white lead paint. × *c.* 80.

Fig. 2. *Phoma violacea* erupting through white lead paint—natural infection. × 10.

Fig. 3. *Phoma violacea* pycnidium (T.S.) erupting through white lead paint.

Fig. 4. Comparison of strains of *Phoma violacea* using the 'painted filter paper' method of testing fungicidal activity of paints. Strains 60, 52, 7, 4, 3, and 1 in vertical rows (left to right). A, control white lead paint. B, DCCAT 1·0%. C, DPMDS 1·0%. D, PMA 1·0%.

Fig. 5. Growth of *Cladosporium herbarum* on a proprietary brand of fungicidal paint. The marked inhibition around the paint test piece is generally characteristic of a fairly common fungicide. Inherently resistant colonies have developed on the paint surface and within the zone of inhibition.

Fig. 6. Painted wooden blocks showing varying degrees of fungal attack after 5 months exposure in glasshouses. A, white lead paint plus PMA 1·0%. B and C, zinc oxide paint—no fungicide. D, white lead paint plus DPMDS 2·0%. E and F, titanium dioxide paint—no fungicide.

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The growth requirements of *Phoma violacea*, with reference to its disfiguration of painted surfaces

By D. E. EVELEIGH*
Department of Botany, University of Exeter

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SUMMARY

Growth requirements of *Phoma violacea* in comparison with the commoner paint-attacking fungi *Pullularia pullulans* and *Cladosporium herbarum* were determined. In liquid culture media, growth occurred over a range of pH values from 3 to 8, double optimal pH values being noted. A wide range of carbohydrates supported good growth. Lipolysis was not marked. *Phoma violacea* strains were autotrophic for vitamins. Zinc oxide exhibited fungistatic properties at 5 % in agar medium, but was not fungicidal. White lead caused slight fungistasis while titanium dioxide had no marked effect on fungal growth.

INTRODUCTION

Occasional reports of the occurrence of *P. violacea* (Bertel) Eveleigh on paint films have been made (Bertel, 1904; Massee, 1911; Haensler, 1921; Nicot-Toulouse, 1953), but no detailed investigations have been made on the physiology of this fungus. An investigation of its nutritional requirements was therefore made while studying the taxonomy of isolates of *Phoma* spp. associated with paint films (Eveleigh, 1961). Comparative studies were made with isolates of two fungi more commonly associated with the degradation of painted surfaces, namely, *Pullularia pullulans* (de Bary) Berkh. and *Cladosporium herbarum* Link (see Table 1).

MATERIALS AND METHODS

Media used in routine experiments were malt agar (Oxoid), a mineral salts medium (MM) consisting of Na_2HPO_4, 0·6 g.; $MgSO_4.7H_2O$, 0·25 g.; NH_4NO_3, 4·0 g.; $FeCl_3$, a trace; glucose, 30 g.; water to 1000 ml.; and a modified Czapek-Dox medium incorporating glucose in place of sucrose. All media were sterilized by autoclaving at 10 lb./sq.in. for 15 min. Detailed results were obtained using the two liquid media described above, the fungi being grown in still culture on 25 ml. of medium in 100 ml. Erlenmeyer flasks. Cultures were incubated at 25° C. for specific periods and the mycelium then filtered on weighed filter papers (Whatman No. 1), washed with distilled water and dried to constant weight by heating at 100° C. Five replicates were used in each treatment and pH values were recorded using a Beckman Cambridge pH meter.

* Present address: National Research Council, Halifax, Nova Scotia, Canada.

PHYSICO-CHEMICAL FACTORS

Temperature. The effect of temperature was considered of particular significance because mould growth on paint is more severe in heated than in unheated glasshouses. Isolates were incubated on malt agar over a range of temperatures and colony diameter used as a measure of growth rate.

Table 1. *Strains of* Cladosporium herbarum *and* Pullularia pullulans *isolated from disfigured paint*

Strain	Substrate	Locality
	Cladosporium herbarum	
C 4	Air isolate	Birmingham
C 29	External paint	Tavistock
C 31	Whitewash on plaster	Exeter
C 58	Paint	Newcastle
C 73	White lead paint	Edinburgh
	Pullularia pullulans	
P 3	Air isolate	Birmingham
P 56	White lead paint	Birmingham
P 58	Paint	Newcastle
P 60	White lead paint	Glasgow
P 69	Paint	Edinburgh

Table 2. *Effect of temperature on growth of 'paint' fungi*

(Diameter (mm.) of colonies at 12 days.)

Strain	5° C.	15° C.	22·5° C.	25° C.	30° C.	37° C.
Phoma violacea 1	+	42	58	39	15	0
P. violacea 3	+	41	53	59	20	0
P. violacea 20	12	43	66	67	12	0
P. violacea 60	+	42	63	65	44	+
Cladosporium herbarum 31	+	27	47	20	10	0
C. herbarum 73	+	5	27	29	16	0
Pullularia pullulans 58	+	21	45	45	29	0
P. pullulans 69	+	40	63	50	20	0

+ Indicates slight growth.

The results showed optima for *Phoma violacea* strains between 22·5 and 25° C. (Table 2). At 30° C. growth was retarded and colonies assumed a small compact dense form which staled relatively early (14 days). Only three out of twenty-eight strains survived incubation at 37° C. for 1 week. A more accurate determination of growth rate was carried out in liquid culture with *P. violacea* strains 1 and 3. The results agreed well with those using colony diameter as a criterion of growth (Text-fig. 1). The optimum temperature for growth of isolates of *Pullularia pullulans* and *Cladosporium herbarum* was slightly lower than that for the *Phoma* isolates, generally being about 22·5° C. (Table 2). *Pullularia pullulans* strains survived incubation at 37° C. for 1 week.

Humidity. The effect of humidity on spore germination of *Phoma* strains was studied by: (*a*) Mounting spores on a cover-slip and inverting over van Tieghem cells

in which the humidity was controlled by use of saturated solutions of various salts (International Critical Tables, 1935); (*b*) Mounting spores on small cellophane squares which had been coated with a 1·0% malt solution and dried, and suspending the inoculated cellophane in jars which contained saturated salt solutions as in (*a*) (Galloway, 1935). Observations were made by mounting the cellophane directly on microscope slides.

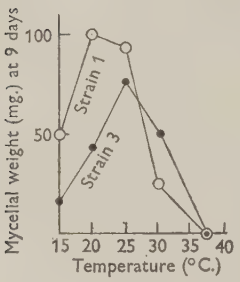


Fig. 1

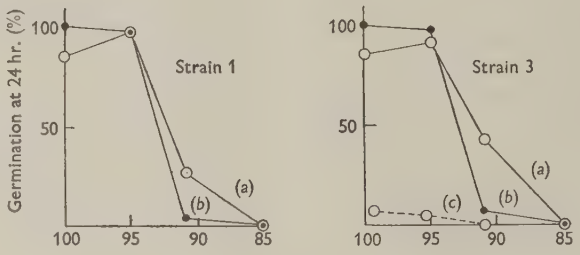
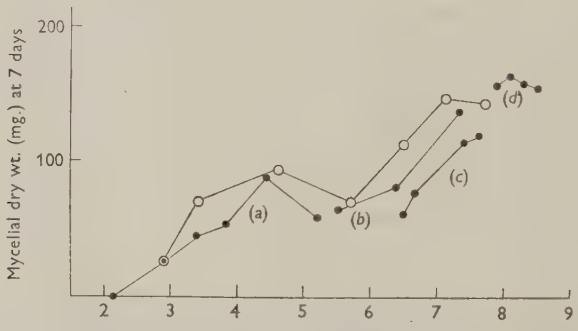


Fig. 2

Text-fig. 1. Effect of temperature on the growth of *Phoma violacea* strains 1 and 3.

Text-fig. 2. Effect of humidity on spore germination of *Phoma violacea* strains 1 and 3. *a* = 30° C.; *b* = 25° C.; *c* = 15° C.



Text-fig. 3. Effect of pH value of medium on the growth of *Phoma violacea*. ○—○ McIlvaine buffers; ●—● Sorensen buffers (*a*, citrate/HCl, *b*, phosphate, *c*, citrate/NaOH, *d*, borate/NaOH).

Both methods showed a high percentage germination above 95% R.H. after 24 hr. at 25° C. using five strains (Text-fig. 2). Germination fell to 5% in all cases at 92% R.H., while no germination occurred after 2 weeks' incubation at 85% R.H. *Cladosporium herbarum* showed a greater percentage germination (10%) at 95% R.H., while a few spores of strains C58 and C73 germinated after 2 weeks' exposure at 85% R.H. Spore germination of all strains investigated fell rapidly even at 98% R.H. at 15° C.

Hydrogen-ion concentration. Growth of *Phoma violacea* was studied over a wide pH range in liquid medium (MM lacking phosphate) incorporating two series of buffers and also a series (MM) using additions of dilute HCl or NaOH to produce the required

pH range. Two buffer series were used (McIlvaine & Sorensen cited by Clarke, 1928), in order to ascertain whether the growth rates observed were due to actual pH or effect of the constituents of the buffer (phosphate or citrate). The buffers were added to 20 ml. of nutrient medium (5 ml. per 20 ml.) each having previously been sterilized separately. In the series carried out using dilute HCl or NaOH the reagents were added before sterilization. Caramelization of glucose occurred in alkaline medium during autoclaving and the pH was rechecked before inoculation.

The results obtained by the different methods (Text-fig. 3) showed close correlation. The wide pH range in which growth occurred is noteworthy and also the appearance of two growth maxima. Similar maxima have previously been reported by Robbins (1924) in *Rhizopus nigricans*, and by Scott (1924) in *Fusarium lycopersici* (cited by Lilly & Barnett, 1951, p. 160). Acid production finally overcame the effect of the buffer present and could be partially responsible for the high growth rates in alkaline medium. It was noted that the colour of the fungal pigment was dependent on pH, changing from red to violet on raising the pH of acidic media.

Table 3. *The effect of light on the growth rate of* Phoma violacea

(Diameter (mm.) of colonies at 10 days.)

Phoma strains	Continuous light	Normal daylight	Darkness
1	17	37	40
2	30	36	38
3	20	38	36
4	22	28	27

Light. Light affected both colony form and pigment production in all the *Phoma* strains. The effect on colony form was investigated by exposing cultures (four strains) on malt agar and Czapek-Dox agar plates to continuous light (using fluorescent-daylight tubes at an intensity of 10 ft. candles per sq.ft.), to normal daylight (September 1957), and to complete darkness, all exposures being at room temperature. Colonies in continuous light grew more slowly than similar colonies in darkness (Table 3), the colony outline in the former changing from the normal smooth outline to an extremely irregular one. Production of pycnidia was unaffected.

Bertel (1904) and Haensler (1921) pointed out independently that light was essential for red pigment production by *P. violacea*. One exception was found to this. Strain No. 2, a mycelial strain and normally an extremely strong pigment producer in the presence of light, produced slight red pigmentation in total darkness. All other colonies grown in the dark became black.

NUTRITIONAL REQUIREMENTS

Carbon. Cladosporium herbarum and *Pullularia pullulans* are known to utilize a wide range of carbohydrates, though *P. pullulans* has little, if any, ability to attack cellulose. There is a lack of data regarding the use of carbohydrates by *Phoma violacea*. The growth of these three genera was studied, using a range of carbohydrates. The carbon sources (2%) were incorporated into Czapek-Dox agar in place of sucrose.

A cellulose medium was prepared by incorporating finely chopped Whatman No. 1 filter papers into Czapek-Dox agar.

All strains of *Cladosporium herbarum* and *Phoma violacea* were able to utilize the carbohydrates tested (Table 4), the polysaccharides being less favourable for growth of the former, while inducing copious pycnidial formation in the latter. Dextrin was utilized more readily than starch, while cellulose permitted only sparse growth, though small pycnidia were formed by the *Phoma* strains. *Cladosporium herbarum* strains did not utilize mannitol well. *Pullularia pullulans* strains were able to utilize most of the carbohydrates tested; growth on xylose and rhamnose was slight and consisted of a few hyphae deep in the agar, while only two strains were able to utilize mannitol. Cellulose in the form of finely chopped filter paper was not utilized by any of the six *Pullularia* strains tested. Growth rates of *Phoma* strains 1, 3 and 4 were measured using liquid media containing various carbohydrates (3%), and similar results were obtained to those using solid media.

Table 4. *Carbohydrate utilization by 'paint' fungi*

(Colony diameter (mm.) after 12 days.)

	Phoma strains						*Pullularia pullulans* P			*Cladosporium herbarum* C		
	1	2	7	52	A	B	56	69	3	31	73	4
Glucose	52	26	50	54	75	55	16	16	16	16	25	25
Mannose	42	25	37	52	49	52	16	20	17	12	18	15
Galactose	50	31	48	45	52	53	12	10	6	21	13	21
Sucrose	60	35	48	55	55	65	20	NT	21	16	18	20
Maltose	45	29	48	48	90	85	18	18	22	22	27	26
Lactose	42	26	50	48	55	76	36	42	37	18	19	21
Raffinose	45	22	48	54	57	85	20	27	22	17	19	22
Xylose	45	35	40	51	61	64	18*	10*	8*	10	9	23
Rhamnose	40	28	31	35	50	58	4*	6*	45*	7	8	18
Mannitol	52	25	49	66	†	85	5*	14*	NT	11	11	20
Dextrin	54	40	52	62	70	85	12*	21*	16*	21	22	25
Starch	47	32	46	52	55	55	7*	11*	18*	11	9	10

* Growth restricted to a few hyphae deep in the agar.
† Spores germinated but no further growth.
NT Not tested.
Phoma A, *P. alternariaceum.*
Phoma B, *P. solanicola.*

Lipolysis was studied as a specialized aspect of carbohydrate metabolism, the question of paint films acting as nutritional substrates having previously aroused much controversy. Lipolytic activity amongst fungi is widespread but is not markedly present in *Cladosporium herbarum*, *P. pullulans*, *Pyrenochaeta terrestris* and *Phoma* spp. (Reese, Cravetz & Mandels, 1955). Lipolytic activities of several 'paint' fungi were compared with certain other fungi using the tributyrin agar test (Oxoid medium PM. 4—Long & Hammer, 1937). Lipolytic activity was assessed by measuring the diameter of the hyaline zone around the colony, taking into account general growth rate of the colony. No pronounced activity was noted, and activity was found to vary amongst strains. *Phoma saprophytica* No. 63 (lipolysis diam. 14 mm., colony diam.

7 mm.), *P. solanicola* (16, 12), and *P. violacea* No. 55 (18, 7) showed greatest activity after 8 days' growth at 25° C. While all *Phoma* 'paint' strains showed slight lipolytic activity certain other *Phoma* spp. often showed generally greater activity, e.g. *P. eupyrena* and *P. glomerata*. *P. glomerata* has been reported occurring on exterior paints in U.S.A. (Drescher, 1958; Rothwell, 1958). The tributyrin agar test indicated that all strains of *Pullularia pullulans* showed only slight lipolytic activity, while that of *Cladosporium herbarum* was even less.

Fatty acids are known to have inhibitory effects on micro-organisms (Karabinos & Ferlin, 1954; Johnson, 1957). The range of fatty acids that could be utilized by the paint isolates under investigation was tested by incorporating the potassium salts of fatty acids as the sole carbon source into a Czapek-Dox salts/agar medium. These salts were prepared by refluxing the respective fatty acids with appropriate amounts of potassium hydroxide in absolute alcohol, followed by precipitation and washing with anhydrous ether, and drying in a desiccator. Alkali-refined linseed oil was also treated similarly. The linoleic acid (B.D.H.-Technical) used was a mixture of linseed oil fatty acids, i.e. linolenic (major) and linoleic acids. The preparation stated above would have converted the linolenic acid to the salt of pseudo-eleostearic acid (10-, 12-, 14-octadecatrienoic acid) due to rearrangement of the double bonds from non-conjugated to conjugated positions (Fieser & Fieser, 1944, p. 399). Sodium ricinoleate was also used in general comparison (B.D.H.-Technical).

Table 5. *Utilization of fatty acids as a carbohydrate source*

(Mean colony diameter (mm.) after 3 weeks.)

	Carbon atoms	*Phoma* strains				*Pullularia pullulans*		*Cladosporium herbarum*	
		7	52	41	26	P 69	P 3	C 31	C 4
Saturated fatty acids (potassium salts)									
Caprylate	8	–	–	–	–	–	–	–	–
Caprate	10	–	–	–	–	–	–	–	–
Laurate	12	–	3	–	–	–	–	–	–
Myristate	14	18	58	34	22	3	6	5	12
Palmitate	16	17	55	32	13	+	4	5	5
Stearate	18	17	58	42	19	8	–	9	11
Unsaturated fatty acids (potassium salts)									
Linoleate*	18	9	38	38	10	5	5	7	9
Ricinoleate†	18	3	5	6	3	–	–		–
Ricinoleate Na	18	17	38	50	21	10	10	7	5
Alkali-refined linseed oil (linoleic + pseudoeleostearic)	18	14	66	40	22	7	10	6	9

\+ Small colony 2–3 mm. diam.
– No growth.
* 'So-called' technical mixed linseed oil fatty acids (B.D.H.).
† 'So-called' technical linseed oil (B.D.H.).

Phoma strains showed sparse flat growth on the higher fatty acids and pigment production was marked. *Cladosporium herbarum* strains showed slow restricted growth while growth of the *Pullularia pullulans* strains was very meagre though not entirely absent. The results are summarized in Table 5. The inability of all three genera to

utilize laurates, caprates and caprylates is a general phenomenon in fungi. Two strains of *P. saprophytica* showed slight growth on laurate.

Haensler (1921) tested lipolytic activity using both wet (unpolymerized) and dry (polymerized) oils on a washed agar medium with and without salts and in all cases without any other carbohydrate being present. He found that *Cladosporium herbarum*, *Alternaria tenuis* and *Phoma* spp. gave good growth responses on dried films only when salts were present. This was confirmed with similar plates using *Phoma violacea* strains 7 and 52, *Cladosporium herbarum* C73, *Pullularia pullulans* P3, and *Aspergillus niger*, the latter included for general comparison (Pl. 1, fig. 1). *Phoma violacea* when grown on these oily substrates formed large oil globules within the hyphae (Pl. 1, fig. 2). Similar results were obtained using polymerized films (dried 14 weeks in a dust-free chamber) of four types of linseed oil—raw, pure boiled, alkali-refined and heat-bodied. *Pullularia pullulans* strains showed the least lipolytic activity. Growth of all genera was extremely sparse on media lacking salts.

Spore germination tests show the same general trend, there being greater germination at the surface of linseed oil in which there had been dissolved Czapek-Dox salts or soil extract. These results confirm the work of Weise (1934) and Findlay (1940), who stressed the importance of other nutrients besides the actual oil. They demonstrated that the residual slime content (the 'foots') of the oil had a beneficial effect on fungal growth.

Nitrogen. The species of 'paint' fungi under investigation are known to have no special nitrogen requirements. Growth on Czapek-Dox agar lacking in sodium nitrate was studied using all strains of *P. pullulans* (17), *Cladosporium herbarum* (22), and *Phoma* spp. (36), incorporating nitrogen into the medium at 0·14% molecular nitrogen. The following substances were used: sodium nitrite, sodium nitrate, ammonium sulphate, ammonium nitrate, urea, casein hydrolysate and peptone. The nitrogen content of the two last-named substances was unknown, and they were used at 3·0 g./l. Sodium nitrite was not assimilated, colonies growing well on most of the other media. Several strains of *Pullularia pullulans* (P 3, 9, 10, 42, 79 and 88) failed to grow on urea, while growth of the other *P. pullulans* strains was poor. A similar experiment in liquid medium with *Phoma violacea* (No. 1) confirmed the results: growth on the nitrate was considerably greater than that found for either of the ammonium substrates.

Vitamins. Liquid culture experiments showed the isolates of the paint fungi to be vitamin-autotrophic. Additions of thiamine (100 μg./l.), pyridoxine (100 μg./l.), nicotinic acid (50 μg./l.) and inositol (100 mg./l.) to a mineral salts medium (MM) failed to stimulate growth of *P. violacea*. Ward (1960) has recently shown a strain of *P. pullulans* to be dependent on thiamine for growth. Four strains of *P. pullulans* (two from Halifax, Canada, and two from Britain, all isolated from paint films) were therefore further studied. Cultures (three replicates) were grown in liquid salts medium (B_1) (Brewer, 1959) with addition of vitamin supplements. Mycelial dry weights were determined by centrifuging the cultures at 3000 r.p.m. for 10 min. in weighed plastic tubes, which were then dried at 100° C. and reweighed. The growth of one strain was stimulated by the addition of thiamine while no effect was noted with the other strains. Results are given in Tables 6 and 7.

Paint pigments. Haensler (1921) and Salvin (1944) demonstrated the fungistatic effect of zinc oxide when employed in high concentrations (5–80 %) in culture media. The former found that the fungistatic effect of white lead varied between genera, having a greater effect on *Aspergillus niger* than on *Pullularia pullulans* and *Cladosporium herbarum.* The effect of white paint pigments (white lead—$2PbCO_3.Pb(OH)_2$, zinc oxide and titanium dioxide) on the growth of *Phoma violacea* was investigated. Various percentages of paint pigments were added aseptically to melted Czapek-Dox agar in flasks, shaken, and the agar poured when near the setting temperature in order to keep the pigments well dispersed in the solid medium. In spite of this precaution, some settling of the relatively heavy pigments occurred and they probably did not

Table 6. *The effect of thiamine*—HCl *on the growth of strains of* Pullularia pullulans

(Mycelial dry wt. mg. at 9 days.)

Strain	Salts medium B_1	Salts medium B_1 plus thiamine—HCl (100 μg./l.)
P 23	263	259
P 24	204	198
P 306	231	222

Table 7. *The effect of vitamins on the growth of* Pullularia pullulans *isolate* P 307

Supplement	Mean mycelial dry wt. (mg.) 6 days	9 days
Control (B_1—25 ml.)	30	247
Thiamine—HCl (100 μg./l.)	243	243
d-Biotin (5 μg./l.)	50	253
Pyridoxine—HCl (100 μg./l.)	66	250
Ca—pantothenate (100 μg./l.)	28	234
All	141	229

exert their total effect at the agar surface. The plates were inoculated with *P. violacea* strain 2. It was seen to react in a similar manner to the moulds tested by Salvin (1944), marked inhibition occurring with 2·5 % zinc oxide but little inhibition with titanium dioxide. The mycelium on agar containing 5·0 % white lead was extremely sparse in comparison with that on 10 % titanium dioxide, although the colony diameters were nearly equal (Table 8).

Liquid culture experiments were used to obtain more precise results. The pigments were added to an inoculated Czapek-Dox medium and incubated in still culture at 25° C. Slight mycelial growth occurred in the presence of white lead and zinc oxide from 0·5 to 10 %. Although these colonies were too small to be measured, they were capable of bringing about changes in the pH of the medium (Table 9). Approximate mycelial weights were determined by dissolving the pigments with nitric acid and filtering the mycelium on to weighed filter papers. Titanium dioxide

could not be removed by this method and the estimation of mycelial growth was made by deducting the original weight of the pigment present from the mycelial dry weight. All grades of zinc oxide used appeared to stimulate growth at 0·25 %. No significant correlation between the amount of growth and particle size of zinc oxide was observed. Salvin (1944) using *Aspergillus niger* and zinc oxide at 0·5 % obtained values slightly below that of the control. He also used a series of zinc oxides with a relatively large

Table 8. *Growth rates of* Phoma violacea (*strain* 2) *in the presence of paint pigments*

(Colony diameter (mm.) after 19 days.)

	Paint pigments		
%	White lead	Zinc oxide	Titanium dioxide
10	45	10	60
5	60	13	60
2·5	62	10	60
0·5	65	20	60
0·25	65	20	60

The pigments were of normal paint grade purity, the titanium dioxide having an anatase crystal structure.

Table 9. *Growth of* Phoma violacea (*strain* 2) *in liquid medium in the presence of paint pigments* (23 *days at* 25° C.)

% pigment		Zinc oxide A	Zinc oxide B	Zinc oxide C	White lead	Titanium dioxide
10	Mean mycelial dry wt. (mg.)	+	+	+	+	++
	Initial pH	7·9	7·9	7·8	8·1	5·8
	Final pH	7·0	7·2	7·1	5·3	3·1
6	Mean mycelial dry wt. (mg.)	+	+	+	+	++
	Initial pH	7·9	7·9	7·9	8·2	5·8
	Final pH	7·1	7·1	7·1	5·5	3·0
3	Mean mycelial dry wt. (mg.)	+	+	+	+	++
	Initial pH	7·8	7·9	7·8	8·1	5·7
	Final pH	7·1	7·1	7·1	5·6	2·7
1·0	Mean mycelial dry wt. (mg.)	+	+	+	+	++
	Initial pH	7·7	7·7	7·7	7·9	5·8
	Final pH	7·1	7·3	7·3	5·7	2·4
0·5	Mean mycelial dry wt. (mg.)	+	+	+	7	42
	Initial pH	7·6	7·7	7·5	7·6	5·8
	Final pH	7·0	7·2	7·4	6·7	2·5
0·25	Mean mycelial dry wt. (mg.)	61	77	59	18	46
	Initial pH	7·1	6·8	7·2	6·7	5·8
	Final pH	4·6	5·1	5·5	4·3	2·3
0·0	Mean mycelial dry wt. (mg.)	46	—	—	—	—
	Initial pH	6·2	—	—	—	—
	Final pH	2·4	—	—	—	—

\+ indicates slight growth.
ZnO A Particle size of B.P. purity.
ZnO B Size larger than paint grade.
ZnO C Size normal paint grade.
TiO_2 and white lead—normal paint grade.

range of particle size and demonstrated that fungistatic activity was dependent on surface area (using colony diameter on agar as a criterion of growth), fungistasis increasing with greater surface area, i.e. small particles were more effective. Titanium dioxide had no effect on mycelial growth of *Phoma violacea* at 0·25 % and 0·5 % and only a slight effect at the higher concentrations tested.

The non-toxic action of the pigments at high concentration was demonstrated by growing *P. violacea*, *Pullularia pullulans* and *Cephaliophora tropica* on malt agar plates in the presence of sterilized muslin 1 in. squares. After 7 days, the 'colonized' muslin squares were transferred to various paint pigments at different concentrations in water, and plated back to malt agar at set intervals. A control experiment was carried out using 1/1000 mercuric chloride solution. 'Squares' were also placed in zinc sulphate solutions (2·5 and 5·0 %). None of the pigments even at 80 % concentration (aq.), nor the zinc sulphate solutions were shown to be toxic after 3 weeks' exposure. Control colonies were all killed after 1 hr. exposure.

DISCUSSION

From this investigation *Phoma violacea* appears as a saprophyte having no specialized requirements for growth, and able to use an extremely wide range of substrates. In the author's experience it is found commonly in heated glasshouses in Britain, the temperature range (15–28° C.) generally encountered being optimum for its growth. Excessive condensation occurs in such situations and physical conditions appear ideal for fungal growth.

Infection is probably transferred by insects but no main source of infection has been revealed. All strains investigated so far have shown no signs of pathogenicity towards plants (Eveleigh, 1961).

Lipolytic activity shown by *P. violacea*, *Pullularia pullulans* and *Cladosporium herbarum* was not marked and was actually less than other comparative species tested, e.g. *Phoma solanicola* and *P. alternariaceum*. These results support the comment referring to a relationship between lipolytic activity and attack of oily substrates that: 'Activity (of fungi) on the oils of paint, or of animal carcasses, evidently hinges on other factors' (Reese, Cravetz & Mandels, 1955). However, the question of whether a direct relationship exists between lipolytic activity of a fungus and its ability to attack paint is itself bound up with the question of whether or not paint films can form a complete nutrient source for fungal growth. Galloway (1955) maintains that paint does not serve as a carbohydrate source to any great degree, nutriment being derived from material below the paint film (e.g. wood) or from surface debris, which would normally be quite sufficient to support growth. The stimulated but limited growth shown by strains of *P. violacea*, *Pullularia pullulans* and *Cladosporium herbarum* on oils to which salts had been added, supports this view.

Zinc oxide is essential as a fungistatic agent in white paints, but its use is precluded in glasshouses because of the phytotoxicity of condensates forming on the paint. White lead paint is therefore still in general use in glasshouses, and this shows greater fungistasis than titanium dioxide paints. The incorporation of fungicides in paints has associated problems, namely attenuation by the drying oils (Leonard & Larson, 1953)

and the phytotoxic action of vapours arising from them in certain instances (Dimond & Stoddard, 1955). Thus, control of fungal growth in internal situations remains difficult.

My thanks are due to Prof. J. Caldwell, Dr G. C. Ainsworth and Dr S. A. J. Tarr for help and advice. Acknowledgement is made to the University of Exeter and Ministry of Education for maintenance throughout the course of this work.

REFERENCES

BERTEL, R. (1904). *Aposphaeria violacea*—ein neuer Glashauspilz. *Öst. bot. Z.* **54**, 205, 233, 288.

BREWER, D. (1959). Physiological studies of *Phialophora fastigiata* and *P. richardsiae*. *Canad. J. Bot.* **37**, 339.

CLARKE, W. M. (1928). *The determination of hydrogen ions*, 3rd. edn. London: Baillière, Tindall and Cox.

DIMOND, A. E. & STODDARD, E. M. (1955). Toxicity to greenhouse roses from paints containing mercury fungicides. *Bull. Conn. agric. Exp. Sta.* no. 595, Sept.

DRESCHER, R. F. (1958). Micro-biology of paint films. IV. Isolation and identification of the microflora on exterior emulsion paints. *Amer. Paint J.* **42**, 80.

EVELEIGH, D. E. (1961). *Trans. Brit. mycol. Soc.* (In the Press).

FIESER, L. F. & FIESER, M. (1944). *Organic Chemistry*. Boston: Heath and Co.

FINDLAY, W. P. K. (1940). Discoloration of paint caused by mould growth and its prevention. *J.O.C.C.A.* **23**, 217.

GALLOWAY, L. D. (1935). The moisture requirements of mould fungi, with special reference to mildew in textiles. *J. Text. Inst.* T 123.

GALLOWAY, L. D. (1955). The growth of moulds on painted surfaces. *J.O.C.C.A.* **38**, 250.

HAENSLER, C. M. (1921). Fungus injury to paint surfaces (Thesis). Rutgers College and the State University, New Jersey.

International Critical Tables of Numerical Data, Physics, Chemistry and Technology. Nat. Res. Coun. of U.S.A. (1933). New York: McGraw-Hill Co.

JOHNSON, G. T. (1957). Fatty acids as carbon sources for the growth of *Spicaria violacea*. *Mycologia*, **49**, 172.

KARABINOS, J. V. & FERLIN, H. J. (1954). Bactericidal activity of certain fatty acids. *J. Amer. Oil. Chem. Soc.* **31**, 228.

LEONARD, J. M. & LARSON, H. L. (1953). Fungicide attenuation by oils. *Naval Res. Lab. Rep.* No. 4228. As a Publication Board Report (PB. NO.) distrib. U.S. Depart. of Commerce, Washington, D.C.

LILLY, V. G. & BARNETT, H. L. (1951). *Physiology of the fungi*. McGraw-Hill Co. Inc.

LONG, H. F. & HAMMER, B. W. (1937). Methods for the detection of lipolysis by micro-organisms. *Iowa St. Coll. J. Sci.* **2**, 343.

MASSEE, G. (1911). A new paint-destroying fungus—*Phoma pigmentivora*. *Kew Bull.* 8, 325.

NICOT-TOULOUSE, J. (1953). Deux *Phoma* isolés sur plâtre humide. *Bull. Soc. mycol. Fr.* **68**, 352.

REESE, E. T., CRAVETZ, H. & MANDELS, G. R. (1955). Activity of fungi on oils. *Farlowia*, **4**, 409.

ROTHWELL, F. M. (1958). Microbiology of paint films. II. Isolation and identification of the microflora on exterior oil paints. *Off. Dig.* **30** (399), 368.

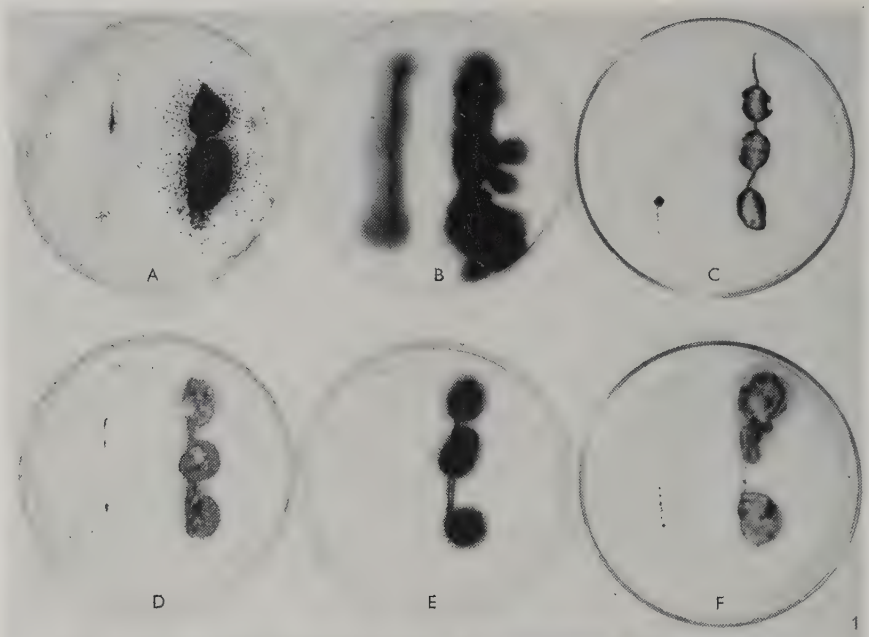
SALVIN, S. B. (1944). Influence of zinc oxide on paint molds. *Industr. Engng Chem.* (*Industr.*), **36**, 336.

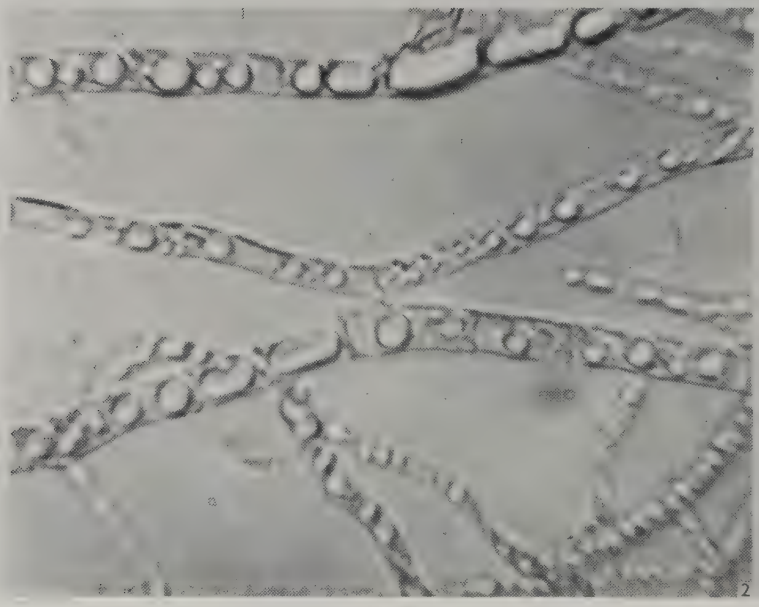
WARD, E. W. B. (1960). Thiamine requirement of *Pullularia pullulans* (de Bary) Berkh. *Canad. J. Bot.* **38**, 891.

WEISE, K. (1934). The breakdown of linseed oil paints by fungi. *Farbenztg.* **34**, 412 and 444.

J.O.C.C.A. Journal of the Oil and Colour Chemists' Association.

Off. Dig. Official Digest of the Federation of Paint and Varnish Production Clubs.





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EXPLANATION OF PLATE

Fig. 1. Growth of fungi on oils. Cultures on a salts agar medium (lacking carbohydrate; washed agar). Streak inoculation directly on agar (left) and on polymerized alkali refined linseed oil (right). A, *Aspergillus niger*; B, *Cladosporium herbarum*; C, *Pullularia pullulans*; D, *Phoma violacea* (No. 52); E, *Phoma violacea* (No. 7); F, *Phoma solanicola*. Control cultures, on oil streaks on agar medium lacking salts, showed very slight growth.

Fig. 2. *Phoma violacea*. Hyphae showing large oil globules after growth on an oil-based medium. × 350.

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Some variants and possible errors in the test-tube dilution and slide-germination methods for laboratory testing of fungicides

By A. H. McINTOSH
Rothamsted Experimental Station, Harpenden, Herts.

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SUMMARY

A method is given for moulding uniform Perspex (polymethyl methacrylate) cavity slides, which are quickly made, almost unbreakable and without joints; they can withstand strong alkalis, but not strong acids or solvents, and are useful for spore germination tests of water-soluble fungicides. When the drops of spore suspension-fungicide mixture in the cavities of these slides or of Böttcher's glass cavity slides are enclosed by cover-slips, there is no meniscus effect, and in tests of long duration the same spores can be counted repeatedly.

In such tests, errors may arise from sorption of the fungicide on the surfaces of pipettes and tubes (dilution stage) and on slides and cover-slips (incubation stage); and from changes in the volume of the drops (incubation stage).

Ions of many metals may be lost by sorption, and the losses are often greater on soft than Pyrex glass. Hg was lost rapidly. In a three-stage serial dilution of $HgCl_2$ solution in soft glassware from 60 to 1·0 p.p.m. of Hg, the loss in strength was 27 %. More Hg was lost from $HgCl_2$ than from phenyl mercuric acetate solutions. The losses on glass or Perspex slides with cover-slips from solutions containing 1·0 p.p.m. of Hg (but no spores in suspension) were 30–35 % in 15 min. and 61–74 % in 24 hr. with $HgCl_2$, but only 5–10 % on glass and 35–37 % on Perspex slides in 24 hr. with phenyl mercuric acetate. Increase in temperature (10–25° C.) slightly increased the loss from $HgCl_2$ but not from phenyl mercuric acetate solutions. Perspex slides, which were used repeatedly for tests of water-soluble mercury compounds and washed in distilled water after each test, eventually became toxic to conidia of *Botrytis fabae* Sardiña.

When the slides, with drops, are placed in moist chambers for incubation, some evaporation is unavoidable because the air close to them is not initially saturated with water vapour. When the chambers were pre-cooled or pre-warmed, so that the temperature of the air in them was uniform, the loss from 0·30 ml. drops in cavity slides was not serious (about 8 %, with or without cover-slips, in 48 hr. at 10 or 25° C.). But when the temperature was not uniform, the possibilities of change in drop volume were much greater: in 48 hr., the volume of open drops could decrease by evaporation (44 % loss at 10° C.) or even increase slightly by condensation (3 % gain at 25° C.). When spores have to be counted on several occasions, the evaporation losses may be more serious.

INTRODUCTION

In the standard slide-germination method for laboratory testing of fungicides (Anon., 1943), and in most modifications of it (Kirby & Frick, 1953; Rabe, 1956), droplets of spore suspensions are placed, for incubation, on glass microscope slides. However, if a cover-slip must be placed over the drop before the spores are counted, they can be counted on only one occasion; and if no cover-slip is used, there is a meniscus effect, which limits the choice of countable spores to those near the centre.

The meniscus effect can be partly overcome by using culture slides (Barratt & Horsfall, 1947) or Böttcher's glass slides, which have circular flat-bottomed cavities (Messrs Griffin and George, Ltd.). This paper describes a method for making somewhat similar cavity slides from Perspex (polymethyl methacrylate) sheet; these, when used with cover-slips, have some advantages over ordinary microscope slides, or Böttcher's glass slides.

Losses by sorption of water-soluble fungicide on the surfaces of pipettes, slides, etc., and by evaporation of water from the drops, may sometimes occur in laboratory tests. Losses by sorption from very dilute aqueous solution are well known in microanalysis; they were followed by measuring the losses in strength of solutions of $HgCl_2$ and phenyl mercuric acetate. The losses by evaporation may be serious when the initial temperature and distribution of water vapour in the moist chambers are not even, and were followed by measuring the increases in strength of dye solutions.

METHODS

Perspex cavity slides

The following method of 'shock moulding' was used to give 6×4 cm. Perspex slides, each with one flat-bottomed circular cavity of diam. 1·4 cm., depth 0·125 cm.

The die and press. The circular die was made from polished mild steel, shaped as shown in the upper part of Fig. 1. The stem was fitted to the upper (moving) jaw of a vertically mounted vice; the lower jaw carried a rigid horizontal base plate, covered by a sheet of asbestos.

Shock moulding. Clean 7×5 cm. rectangles, cut from $\frac{1}{4}$ in. Perspex sheet, were dusted with chamois leather, heated in batches on an enamel tray in an oven at 150° C. for at least 30 min., transferred singly to the vice on the (cool) metal transfer plate (Fig. 1), pressed for 2 min., and the pressure-deformed sides were trimmed to perpendicular in a lathe-saw, so that they could be held on a microscope stage.

I am indebted to Mr W. E. Frost, Plastics Division, I.C.I. Ltd., for the following notes on pre-heating. To prevent over-heating, the tray holding the Perspex should be cork-insulated from the metal structure of the oven; and, for other thicknesses of sheet, the minimum heating time (min.) should be $10 + (80 \times \text{thickness in in.})$.

Use of slides in spore germination tests. The inner cavities of these slides, which were of uniform depth, have been satisfactorily used, along with the test-tube dilution method (Anon., 1947), for tests of water-soluble fungicides on conidia of some strains of *Botrytis fabae* Sardiña.

The nominal volume of the inner depression, calculated from the dimensions of the

die (Fig. 1), was 0·19 ml. In practice, 0·25 ml. of the spore suspension-fungicide mixture was placed in the inner cavity, from which it did not overflow, and the lens-shaped drop was at once covered with a new polished round $\frac{7}{8}$ in. cover-slip. The liquid spread to the edge of the cover-slip, but no further (Fig. 2).

After incubation in moist chambers, the conidia (about $14 \times 8 \mu$), were counted by traversing the inner cavity on a microscope stage ($\times 80$). Two parallel traverses were made per cavity, one on each side of and about half a radius from the centre, where spores often formed clusters. All the single spores in each traverse were counted; with a spore concentration of about 6500/ml., this gave 100–120 single spores per traverse. Germination of conidia of *B. fabae*, as measured in this way in $1:10^4$ orange juice, was regularly 90% or more after 24 hr. at 25° C.

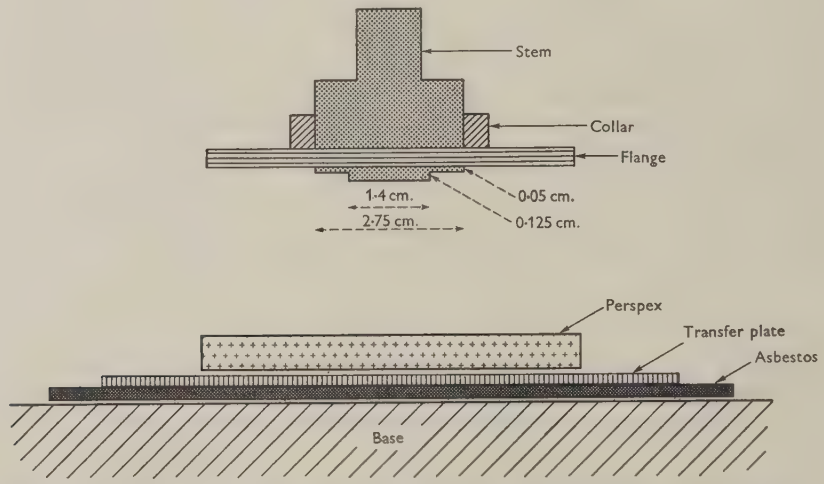

Fig. 1. Sectional view of die, Perspex etc., before shock moulding; vice not shown.

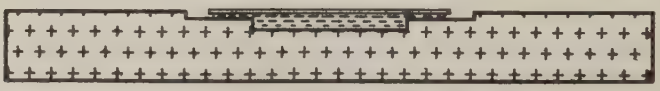

Fig. 2. Sectional view of Perspex cavity slide, with drop and cover-slip in position.

Cleaning. At the end of each test, the cover-slips were removed, and the surfaces of the inner and outer cavities were swabbed with wet cotton wool to remove fungal material; the slides were washed in running tap water overnight, soaked in three changes of distilled water at 70° C. for 10 min. each, and dried at 60–65° C.

Perspex easily acquires a static electric charge and attracts dust, and so the slides were either used as soon as possible after removal from the drying oven, or were stored in a closed box, rewashed once in distilled water at 70° C. for 10 min., dried and used.

Losses on surfaces

Test-tube dilution stage ($HgCl_2$ *only*). The following procedure was used as an arbitrary standard dilution sequence; it differs in detail from the standard test-tube dilution method (Anon., 1947), but is similar to the routine used in my spore germination tests (McIntosh, 1961).

The tests were done at constant temperature; all water and glassware (which, except for the graduated flasks, was of soft glass) was pre-cooled or pre-warmed to 10 or 25° C. before each test. From a freshly made solution (S) of $HgCl_2$ in distilled water (60 p.p.m. of Hg) in a 500 ml. borosilicate graduated flask, 1·0 ml. was transferred in a bulb pipette to 9·0 ml. distilled water in a $\frac{3}{4}$ in. test-tube, shaken and left for 5 min. (nominal concentration 6·0 p.p.m. of Hg). 5·0 ml. of this was transferred in a 10 ml. graduated pipette to 5·0 ml. of distilled water in a second tube, shaken and left for 5 min. (nominal concentration 3·0 p.p.m. of Hg). 1·0 ml. of this was transferred in a bulb pipette to a third tube containing 2·0 ml. of distilled water, shaken and left for 5 min. (nominal concentration 1·0 p.p.m. of Hg). 2·5 ml. of this were removed in a bulb pipette and combined with 2·5 ml. from another identical dilution, for analysis by the dithizone method (Sandell, 1959). The expected amount in each 5·0 ml. sample was 5·0 μg. of Hg. Direct dilution of 1·67 ml. of the original solution S (in a 2 ml. graduated pipette) to 100 ml. (in a graduated flask) gave a check on its concentration.

Between tests, all glassware was soaked in 1 % HNO_3 for 1 hr., washed overnight in tap water (pipettes), or for 30 min. in boiling distilled water (tubes) and dried at 150° C.

Cavity slides. All materials were pre-cooled or pre-warmed to the temperature of the test; all pipettes, tubes and Böttcher's slides (diam. 1·5 cm., depth 0·2 cm.) were of soft glass.

Drops of solutions of $HgCl_2$ (5·0 or 1·0 p.p.m. of Hg) or phenyl mercuric acetate (1·0 p.p.m. of Hg) were placed in the cavities of Perspex (0·25 ml.) or Böttcher's slides (0·41 ml.) from a 1·0 ml. graduated pipette, and were covered with new polished round or square $\frac{7}{8}$ in. cover-slips. The drops were left for 15 min. at room temperature (about 18° C.), or for 24 hr. in moist chambers in constant temperature rooms at 10 or 25° C. The drops were then taken off the slides with a small pipette and combined in a test-tube until there was enough for analysis of the remaining mercury (1·5 ml. of the 5·0 p.p.m. solution, and 5·0 ml. of the others).

Analyses were by the dithizone methods of Sandell (1959) for $HgCl_2$, and Miller, Polley & Gould (1951) and Clifford (1938) for phenyl mercuric acetate. Direct analysis of samples of the original solutions gave checks on their concentrations.

Between tests, Perspex slides were soaked in 1 % HNO_3 for 1 hr., and then washed as on p. 426; Böttcher's slides and glassware were cleaned as above.

This method gave figures for the combined losses on slides, cover-slips, pipettes and tubes.

Evaporation

Evaporation of water (from Böttcher's glass slides, diam. 1·5 cm., depth 0·1 cm.) was followed by measuring the increases in concentration of solutions of the water-soluble dye Naphthalene Scarlet 4RS, which is not sorbed by glass, and is stable in solution.

0·30 ml. of a 0·002 % dye solution was placed in each cavity, and the slides, with their drops covered in some series by new polished square $\frac{7}{8}$ in. cover-slips, were placed in 30 × 10 cm. moist chambers, with about twenty slides per chamber; the upper surfaces of the slides were about 0·5 cm. above the surface of the water in the seal, and the roofs of the chambers about 1·0 cm. above the slides.

After 48 hr., the drops from fifteen to twenty slides, which together gave about 2·5 ml., were combined in a colorimeter tube, and the optical density measured at 510 mμ; the percentage evaporation was calculated from the increase in concentration.

RESULTS

Losses on surfaces

Test-tube dilution stage. The mean figures, from nine samples at each temperature, for μg. of Hg remaining in 5·0 ml., were 3·68 ± 0·31 at 10° C. and 3·63 ± 0·38 at 25° C.; the loss was evidently unaffected by temperature. The mean figure for both temperatures (3·66 ± 0·35) is 73 % of the expected value (5·0); the corresponding figure from six samples of directly diluted solution was 4·93 ± 0·27, i.e. 99 % of the expected value.

Table 1. *Amounts of* Hg *recovered from drops of aqueous solution on glass and Perspex cavity slides, with cover-slips. For details of methods, see p.* 427

Slides	Surface area and volume of drops (cm.²/ml.)	Temp. (° C.)	Conc. of Hg (p.p.m.)	Expected Hg (μg.)	μg. of Hg found per sample (with numbers of samples) of solutions of $HgCl_2$ after 15 min.	$HgCl_2$ after 24 hr.	Phenyl mercuric acetate after 24 hr.
Glass	10·62/0·41	25	1·0	5·0	—	1·45 ± 0·13 (4)	4·77 ± 0·06 (3)
		18	1·0	5·0	3·48 ± 0·34 (8)	—	—
		10	1·0	5·0	—	1·93 ± 0·33 (4)	4·50 ± 0·10 (3)
Perspex	8·15/0·25	25	5·0	7·5	—	3·73 ± 0·55 (12)	—
			1·0	5·0	—	1·28 ± 0·52 (4)	3·23 ± 0·25 (3)
		18	1·0	5·0	3·24 ± 0·40 (8)	—	—
		10	5·0	7·5	—	4·39 ± 0·53 (12)	—
			1·0	5·0	—	1·58 ± 0·50 (4)	3·13 ± 0·06 (3)
Direct analysis		18	5·0	7·5	—	7·50 ± 0·24 (12)	—
			1·0	5·0	4·94 ± 0·11 (15)	4·75 ± 0·09 (12)	4·89 ± 0·11 (13)

Cavity slides. Table 1 summarizes the results. The figures for 'direct analysis' were obtained from the original solutions after they had stood in 100 ml. borosilicate graduated flasks for 15 min. or 24 hr. at room temperature. The other figures are the

amounts of Hg remaining after the losses on the slides, cover-slips, pipettes and tubes. The separate losses were not found, but it is assumed that the greatest loss was on the slides and cover-slips. The figures are not corrected for losses by evaporation (see below).

The specific surface of the drops on the glass slides (25·9 cm.²/ml.) was less than on the Perspex slides (32·6 cm.²/ml.), so the two sets of figures are not exactly comparable. However, it is clear that whereas much the same amount of Hg was lost from $HgCl_2$ solutions on each surface, more Hg was lost from phenyl mercuric acetate solutions on Perspex than on glass slides. Losses were much greater from $HgCl_2$ than from phenyl mercuric acetate solution on both surfaces. Temperature had no significant effect on the losses from phenyl mercuric acetate solution on either glass or Perspex, but increase in temperature from 10 to 25° C. slightly increased the losses from $HgCl_2$ solutions on both surfaces. Not surprisingly, the percentage loss of Hg (from $HgCl_2$ solutions on Perspex) increased with increasing dilution; the mean percentages of Hg lost in 24 hr. from the stronger and weaker solutions were 41 and 68 % at 10° C. and 50 and 74 % at 25° C. About half of these losses, from the weaker solution, occurred within the first 15 min. on both glass and Perspex.

Evaporation

When the solutions, pipettes, slides, moist chambers and the water in them were pre-cooled or pre-warmed in constant-temperature rooms at 10 or 25° C., the temperature of the air in the moist chambers was uniform, but the relative humidity of the air near the drops was initially less than 100 %, and some evaporation from the drops was unavoidable. Its extent was the same at each temperature, whether cover-slips were used or not; the mean figure for the percentage evaporating in 48 hr. from 0·30 ml. drops was 7·8 ± 3·2 % (37 measurements).

Table 2. *Percentages of water lost by evaporation from or gained by condensation* on 0·30 ml. drops in Böttcher's slides in 48 hr. (with numbers of measurements). For details of treatments (a) and (b), see pp. 429–30.*

Temp. (° C.)	Surfaces of drops	Treatment (*a*)	Treatment (*b*)
25	With cover-slips	3·4 ± 3·6 (5)	—
	Without cover-slips	3·4* ± 1·5 (5)	5·5 ± 1·8 (8)
10	With cover-slips	—	25·5 ± 4·1 (4)
	Without cover-slips	—	44·0 ± 5·8 (5)

However, under other conditions, the movement of water vapour was affected by the cover-slips, and by the incubation temperature.

(*a*) Some moist chambers, which had damp blotting paper on the undersides of their roofs, were pre-warmed, and incubated with slides and drops in a roughly 25° C. room, in which the heating was above bench level. After the unavoidable initial evaporation from the drops, water vapour from the moist air near the tops of the chambers, which were slightly warmer than the lower parts, condensed on the drops without cover-slips; but when cover-slips were used, the net effect was very slight evaporation (Table 2).

(*b*) Moist chambers, charged with slides and drops at room temperature, but without blotting paper on their roofs, were at once put on the floors of cabinets at 10 or 25° C. The tops of the chambers at 10° C. were, initially at least, cooler than the rest, and water vapour from the drops condensed on the roofs of the chambers. The loss at 25° C. was small, but at 10° C. it was much larger, especially when no cover-slips were used (Table 2).

DISCUSSION

Cavity slides. The Perspex slides used in the tests described here were pre-heated to 170° C. before shock moulding; this may have caused some depolymerization, and increased the uptake of Hg. However, losses of Hg from $HgCl_2$ solutions on slides pre-heated to only 150° C. (at which there is little or no depolymerization) were of the same order as those in Table 1. Losses on slides made from polystyrene sheet (from Messrs Erinoid, Ltd., Stroud) were equally high; these slides are more difficult to make, and are not recommended.

The Perspex slides have a limited usefulness, as they cannot be cleaned with strong acids, or with many organic solvents. However, they are not attacked by strong alkalis, and for some purposes they have advantages over conventional glass slides.

Unlike Böttcher's glass slides, whose dimensions can vary from batch to batch, the Perspex slides have cavities of uniform size; and they are quickly made, almost unbreakable and without joints. When cover-slips are used, there is no meniscus effect, so that all the spores in a traverse can be counted, without eye-strain from the shaking drop, and without subjectivity in the choice of spores to be counted; and gross evaporation is decreased.

Some of these advantages are shared by Böttcher's glass slides; both types can be used in tests of long duration, in which the spores are counted repeatedly.

Sorption. The sorption losses of ions on glass or Perspex resemble the losses of Hg from phenyl mercuric acetate solution on the surfaces of soil, metals, wood, etc. (Miller, Gould & Polley, 1957); and the smaller losses of DDT from dilute aqueous suspensions or solutions on glass, aluminium or paper (Bowman, Acree, Schmidt & Beroza, 1959; Weidhaas, Schmidt & Bowman, 1960).

Glass surfaces can sorb many ions, including even sodium, from aqueous solution (Schoonover, 1935; Kellogg, 1945; Hensley, Long & Willard, 1949; Hensley, 1951). Sorption losses can cause serious errors in micro-analysis, particularly of heavy metals; they almost certainly occur with all the fungitoxic metals (and possibly with other water-soluble fungicides). They are well known with silver and mercury (Freundlich & Söllner, 1928; Stock & Neuenschwander-Lemmer, 1938; Sandell, 1959). The losses, or rates of loss, increase with temperature (Kolthoff, 1921; Schoonover, 1935; Hensley *et al.* 1949; Hensley, 1951); the percentage loss increases with the degree of dilution (Stock & Neuenschwander-Lemmer, 1938; Hensley, Long & Willard, 1949; Schönfeld & Broda, 1951); and, at least with salts of silver and mercury, is less on Pyrex than on soda glass (Schoonover, 1935; Sandell, 1959). The rates of release of some sorbed ions into distilled water increase with temperature (Dwyer, 1938; Hensley *et al.* 1948).

The increased percentage loss at higher temperatures and dilutions is confirmed

here. The losses are obviously not the cause of the increased fungitoxicity of mercury salts at higher temperatures (McIntosh, 1961). The lower concentration (1·0 p.p.m. of Hg) in my tests was decided by the lower limit of the dithizone method of analysis. In spore-germination tests, the concentration may often be less than this, and the possible percentage losses correspondingly larger. Although the losses at the dilution stage may be decreased by using Pyrex glassware, some losses are inevitable. Large surfaces are involved; the inner surface area of a 1 ml. bulb pipette, for example, is about 15 cm.^{2}. The percentage of fungicide lost by sorption may not be very great in the highest concentration used, but it increases progressively with each step of the serial dilution. The effect of this is to make the slope of the probit line artificially high (Finney, 1952; Horsfall, 1956). In comparative tests of the same fungicide this may not matter, but the advantage of standardizing the dilution sequence is obvious.

Losses of mercury at the dilution stage, although smaller in my tests than on the slides, are likely to be more serious in practice. Spores can take up large amounts of many chemicals, including mercury, from solution within a few minutes (McCallan & Miller, 1958), and are usually added to the diluted fungicide solutions in the test tubes. At this stage, the specific surface of the spore suspension-fungicide mixture is much smaller than that of the drops on the slides, and the spores may take up most of the available toxicant before the drops are put on the slides. My tests do not show the distribution of mercury between container surfaces and spores; but it is clear that not all of the metal is taken up and retained by the spores. Perspex slides, which had been repeatedly used for tests of water-soluble mercury compounds and cleaned as on p. 426, eventually became toxic to conidia of *B. fabae*.

In general, the proportion of fungicide lost on the slides will depend on the time passing between adding the spores to the solutions and putting the drops on the slides, as well as on the relative rates of uptake of poison by container surface and spores. Copper, zinc and cadmium are taken up by spores, from solutions of their salts, much more slowly than silver and mercury (Miller & McCallan, 1957), but are nevertheless sorbed by glass (Kolthoff, 1921; Dwyer, 1938). In tests of these metals, and possibly of other toxicants, losses on the slides may be as large as or larger than at the dilution stage.

Evaporation. In many testing routines, the drops are smaller (about 0·05 ml.) than in my tests (0·25–0·41 ml.), and the percentage losses by evaporation may be correspondingly larger.

The unavoidable initial loss, which occurs before the air in the moist chambers is uniformly saturated with water vapour, was not serious under commonly used conditions and, when the spores are counted on only one occasion, may be taken as a systematic error. However, in tests of long duration, when the moist chambers are often opened for counting, the loss by evaporation from uncovered drops may be more important. When the temperature of incubation is much below room temperature, losses need not be large if all the materials are pre-cooled.

I would like to thank Messrs I.C.I. Ltd. for a sample of Naphthalene Scarlet 4RS.

REFERENCES

ANON. (1943). The slide-germination method of evaluating protectant fungicides. *Phytopathology*, **33**, 627.

ANON. (1947). Test tube dilution technique for use with the slide-germination method of evaluating protectant fungicides. *Phytopathology*, **37**, 354.

BARRATT, R. W. & HORSFALL, J. G. (1947). Fungicidal action of metallic alkyl bisdithiocarbamates. *Bull. Conn. agric. Exp. Sta.* no. 508.

BOWMAN, M. C., ACREE, F., SCHMIDT, C. H. & BEROZA, M. (1959). Fate of DDT in larvicide suspensions. *J. econ. Ent.* **52**, 1038.

CLIFFORD, P. A. (1938). Some notes on the stability of dithizone solutions. *J. Ass. off. agric. Chem., Wash.*, **21**, 695.

DWYER, F. P. (1938). The adsorption of diazo compounds on cadmium and magnesium hydroxides. Part 6—The adsorption of cadmium ions on solid surfaces. *J. Aust. chem. Inst.* **5**, 338.

FINNEY, D. J. (1952). *Probit analysis*, 2nd. ed. Cambridge University Press.

FREUNDLICH, H. & SÖLLNER, K. (1928). Zur Erklärung der oligodynamischen Wirkung. *Biochem. Z.* **203**, 3.

HENSLEY, J. W. (1951). Adsorption of tagged phosphate ions on glass surfaces as related to alkaline attack. *J. Amer. ceram. Soc.* **34**, 188.

HENSLEY, J. W., LONG, A. O. & WILLARD, J. E. (1948). Reactions of ions in aqueous solution with glass and metal surfaces. *J. Amer. chem. Soc.* **70**, 3146.

HENSLEY, J. W., LONG, A. O. & WILLARD, J. E. (1949). Reactions of ions in aqueous solution with glass and metal surfaces. Studies with radioactive tracers. *Industr. Engng Chem.* (*Industr.*), **41**, 1415.

HORSFALL, J. G. (1956). *Principles of fungicidal action*. Waltham, Mass.: Chronica Botanica Co.

KELLOGG, H. H. (1945). The use of contact angles for the study of adsorption on glass surfaces. *Glass Science Bulletin*, **2**, 58. Abstract in Weyl, W.A., *Glass Ind.* **28**, 231 (1947).

KIRBY, A. H. M. & FRICK, E. L. (1953). Modifications in the glass-slide spore-germination test for use in fungicide research. *Rep. E. Malling Res. Sta. for* 1952, p. 161.

KOLTHOFF, I. M. (1921). The importance of adsorption in analytical chemistry. IX. Glasswool as a filter material. *Pharm. Weekbl.* **58**, 463. (*J. chem. Soc.* **120**, ii, 409; *Chem. Abstr.* **15**, 3041.)

MCCALLAN, S. E. A. & MILLER, L. P. (1958). Innate toxicity of fungicides. *Advanc. Pest Control Res.* **2**, 107.

MCINTOSH, A. H. (1961). Effects of time and temperature on the fungistatic action of mercury-containing and other compounds on conidia of *Botrytis fabae* Sardiña. *Ann. appl. Biol.* **49**, 433.

MILLER, L. P. & MCCALLAN, S. E. A. (1957). Toxic action of metal ions to fungus spores. *J. agric. Fd Chem.* **5**, 116.

MILLER, V. L., GOULD, C. J. & POLLEY, D. (1957). Some chemical properties of phenylmercury acetate in relation to fungicidal performance. *Phytopathology*, **47**, 722.

MILLER, V. L., POLLEY, D. & GOULD, C. J. (1951). Phenyl mercuric or ethyl mercuric compounds. Direct determination of several compounds in dilute aqueous solution. *Analyt. Chem.* **23**, 1286.

RABE, W. (1956). Studies on the action of wettable sulphur against conidia of *Venturia inaequalis* (Cke.) Wint. *Höfchenbr. Wiss.* **9**, 1.

SANDELL, E. B. (1959). *Colorimetric determination of traces of metals*, 3rd edn. New York: Interscience Publishers.

SCHÖNFELD, T. & BRODA, E. (1951). Ionenadsorption an Papier- und Glasoberflächen. *Mikrochemie*, **36/37**, 537.

SCHOONOVER, I. C. (1935). A colorimetric method for the quantitative determination of small amounts of silver by use of *p*-dimethylaminobenzalrhodanine. *J. Res. nat. Bur. Stand.* **15**, 377.

STOCK, A. & NEUENSCHWANDER-LEMMER, N. (1938). Die mikroanalytische Quecksilberbestimmung (XXVII. Mitteil. über Wirkung und Verbreitung des Quecksilbers). *Ber. dtsch. chem. Ges.* **71**, 550.

WEIDHAAS, D. E., SCHMIDT, C. H. & BOWMAN, M. C. (1960). Effects of heterogeneous distribution and codistillation on the results of tests with DDT against mosquito larvae. *J. econ. Ent.* **53**, 121.

Ann. appl. Biol. (1961), **49**, 433–444
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Effects of time and temperature on the fungistatic action of mercury-containing and other compounds on conidia of *Botrytis fabae* Sardiña

By A. H. McINTOSH
Rothamsted Experimental Station, Harpenden, Herts.

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SUMMARY

The *in vitro* fungistatic action of nineteen compounds was measured on conidia of two strains of *Botrytis fabae* Sardiña in slide-germination tests, at two or three incubation temperatures in the range 10–25° C.; the effects were assessed at intervals up to 14 days from the start of each test.

In general, the ED 50's increased as incubation time passed, finally approaching or reaching steady values. With each compound, an increase in incubation temperature increased the speed of action (i.e. decreased the time taken for the ED 50 to reach a steady value), and increased the ultimate toxicity. Temperature coefficients were calculated as ratios of ED 50's and were 'positive' or 'negative' according to whether the toxic action was greater or less at the higher temperature.

The conidia of the two strains of *B. fabae* behaved similarly, but the temperature coefficients were larger with one strain (A) than the other (B).

Mercuric cyanide, chloride, bromide, acetate and thiocyanate gave positive coefficients which increased in size as incubation time passed. In these tests, which lasted for only 3 days, the largest 10:25° C. coefficient (+35) was given by the cyanide after 3 days (strain A); the corresponding figures for the chloride and bromide were +10·5 and +6·0. Phenyl mercury acetate, methyl mercury nitrile, silver nitrate, copper sulphate, 8-hydroxyquinoline, acetamide, *p*-nitrophenol, 2,4-dinitrophenol and pentachlorophenol gave coefficients which were usually small and positive (about +1·3), and changed only slightly in size as incubation time passed. *t*-Butyl alcohol, pyridine, 3-picoline, phenol and *p*-chlorophenol gave coefficients which changed from negative (−3·9 to −1·5) to positive (+1·3 to +3·2) after 2–7 days (strain B).

There was little evidence that spores are more resistant to fungicides at their optimum temperature for germination, i.e. the temperature at which maximum germination is reached soonest (15–20° C. for *B. fabae*).

Mercuric cyanide, chloride and bromide, which are scarcely ionized in aqueous solution, also resembled non-electrolytes in that the sizes of their positive temperature coefficients were inversely related to their solvent:water partition coefficients, i.e. the temperature coefficients increased with decrease in the presumed rates at which lipoid membranes are penetrated by diffusion. This correlation did not extend to other compounds.

INTRODUCTION

This paper gives the results of *in vitro* measurements of the effects of incubation temperature and the passage of incubation time on the fungistatic action of water-soluble compounds on conidia of *B. fabae* Sardiña. The effects of temperature on retention of fungicide by surfaces, on weathering and volatility of deposits, on solubility and rate of solution of fungicide in spore droplets, etc., were not involved.

MATERIALS AND METHODS

Table 1 lists the compounds used. Some of them, obtained as laboratory reagent quality, were purified as follows: *t*-butyl alcohol was dried over lime, and filtered (Young & Fortey, 1902); 8-hydroxyquinoline sulphate was recrystallized from ethyl alcohol; pentachlorophenol (sodium salt) was recrystallized from petroleum ether (80–100° C.) containing 10% acetone, with charcoal; 3-picoline was redistilled.

Table 1. *Compounds used.* L = *laboratory reagent;* P = *laboratory reagent, purified* (*see text*)*;* A = *analytical or other highly purified quality*

Compound	Quality	m.p. (corr.) or b.p. (° C.)
Acetamide	L	77–79
t-Butyl alcohol	P	—
Copper sulphate	A	—
8-Hydroxyquinoline sulphate	P	163–168
Mercuric acetate	L	—
Mercuric bromide	L	—
Mercuric chloride	A	—
Mercuric cyanide	L	—
Mercuric thiocyanate	L	—
Methyl mercury nitrile	L	90–91
Phenyl mercury acetate	A	151–152
Phenol	A	40–41
Phenol, *p*-chloro	L	34–37
Phenol, *p*-nitro	A	112–113
Phenol, 2,4-dinitro	L	110–111
Phenol, pentachloro (sodium salt)	P	—
Pyridine	A	—
Pyridine, 3-methyl (= 3-picoline)	P	140–141 (b.p.)
Silver nitrate	A	—

The test organisms were two freely sporing strains of *B. fabae*: strain A (IMI Herb. no. 79681), and strain B, obtained as a culture on potato dextrose agar from Dr R. J. W. Byrde, Long Ashton Research Station, Bristol.

The tests on strain A, which were mostly of heavy-metal salts, were done in distilled water (pH 5·2). With strain B, salts and non-ionizing compounds were tested in distilled water; acidic and basic compounds were tested in 0·05 M phthalate buffer (pH nominally 4·0). Both media contained $1:10^4$ freshly filtered orange juice.

The strains were grown on medium X of Last & Hamley (1956); inoculum for sub-cultures was taken from 2-week-old cultures. Conidia, taken from 11- to 17-day-old cultures, were washed twice in distilled water by centrifuging, and suspended in dis-

tilled water at 20,000 conidia/ml. (tests in distilled water), or 40,000 conidia/ml. (tests at pH 4·0).

Spore germination tests followed the general design recommended by the American Phytopathological Society (Anon., 1943, 1947); for details not given in these papers or below, see McIntosh (1961).

All glassware, solutions, slides, moist chambers, etc., were pre-cooled or pre-warmed to the incubation temperature. Compounds were serially diluted, at each temperature, in test-tubes; the dose ratio was usually 1·67, except in tests of mercuric cyanide, which gave much flatter dose-response lines (Horsfall, 1956) than the other compounds. One ml. of each concentration was added to a clean test-tube containing either a mixture of 1 ml. spore suspension and 1 ml. of 1:3333 orange juice solution (tests in distilled water), or a mixture of 3 ml. of 0·1 M potassium hydrogen phthalate solution, 1 ml. of spore suspension and 1 ml. of 1:1667 orange juice solution (tests at pH 4·0). Drops of the final mixtures were incubated in Perspex cavity slides (0·25 ml.) or Böttcher's glass cavity slides (0·30–0·41 ml.) with cover-slips, at 10, 20 and 25° C. (strain A) or 10 and 25° C. (strain B).

Germinated and ungerminated conidia were counted after incubation for 1, 2 and 3 days (strain A), or for various times up to 14 days (strain B). In each test there were 100–120 conidia per traverse, two traverses per slide, and one slide (strain A) or two slides (strain B) per concentration of compound. The angles (Knudsen & Curtis, 1947; Finney, 1952*a*) corresponding to the percentages ungerminated in the combined replicate traverses were plotted against logarithms of molar concentrations, and the log ED 50's (Finney, 1952*b*) were read from the lines fitted by eye to the 3–6 points obtained. There were four replicates (strain A) or two replicates (strain B) of each test. The mean log ED 50's given below are each based on about 4000 conidia; their approximate confidence limits were calculated as twice the standard errors, which were found by the method of Miller & Tainter (1944) and formula 3·7 of Finney (1952*b*), from the combined results from the replicate tests. 'Temperature coefficients' of toxic action were calculated as the ratios of the antilogarithms of mean log ED 50's. Numerical values for temperature coefficients are preceded by + or −, according to whether the toxic action was greater or less at the higher temperature. This method has also been used for expressing temperature coefficients of insecticides (Das & McIntosh, 1961). A *t*-test was used to assess the significance of the differences between mean log ED 50's; the standard errors of the mean log ED 50's were so small that a temperature coefficient of 1·1 or even less often corresponded to a significant effect.

PHYSICAL PROPERTIES OF COMPOUNDS

Some physical properties are quoted in the Discussion; the sources of these figures are now given.

The pH's of the final mixtures were, in the tests in distilled water, 5·2 (heavy-metal salts, organo-mercury compounds, *t*-butyl alcohol, 8-hydroxyquinoline and acetamide) and 5·4–5·8, depending on the concentration (pentachlorophenol); similarly, in the test in 0·05 M phthalate buffer, 5·3–6·0 (pyridine), 4·6–6·1 (3-picoline), and 4·0 (phenol and other substituted phenols). The percentages ionized at these pH's at

room temperature were calculated from figures given, for mercury salts, by Ephraim (1948), Sidgwick (1950), and Long & Kobe (1951); for phenyl mercury acetate, by Jensen (1946); for pentachlorophenol, by Blackman, Parke & Garton (1955), and for other compounds, by Heilbron & Bunbury (1953). According to a letter from the Chipman Chemical Co., methyl mercury nitrile is not ionized in solution. The effects of temperature on ionization are, in general, slight.

Values for solvent:water partitition coefficients are approximate for 18–25° C., and are mostly based on published figures. When the partition coefficient varies with concentration, the figure for the concentration closest to the ED 50 at 25° C. is given. Direct determinations are quoted when possible; otherwise, the ratios of solubilities in g./100 ml. of solution, or failing that, g./100 ml. of solvent, are given. Many but not all of the figures are taken from *Beilstein* and from Seidell's *Solubilities*. For mercuric halides, cyanide and thiocyanate, see also Marcus (1957); for phenyl mercury acetate, see Miller & Polley (1954); for phenols, acetamide and pyridine, see also Speyers (1902), Carswell & Hatfield (1939), Macy (1948), Collander (1949), Blackman *et al.* (1955), and Sandell (1958). A few unpublished figures for phenyl mercury acetate and methyl mercury nitrile were obtained directly from other laboratories; and a few solubilities were specially measured.

Acetamide is hydrolysed in aqueous solution, but the reaction is very slow; the half-life is about 11 days in 0·1 M HCl at 25° C. (Reitz, 1939).

RESULTS

Conidia of both strains germinated well in the range 5–29° C. in distilled water or in 0·05 M phthalate buffer (pH 4·0). However, at four extreme temperatures (5, 10, 25 and 29° C.), germination was about 6% higher in distilled water than in buffer. In addition, germination was often incomplete after incubation for only 1 day at these extreme temperatures (e.g. 1% at 5° C. and 88% at 10° C., in buffer); however, it increased to about 97% at all four temperatures when the incubation time was increased to 2 days. At 15 and 20° C., germination was about 99% after only 1 day. There was no germination at 33° C. Thus the optimum temperature for germination, or temperature at which maximum germination was reached soonest (Wellman & McCallan, 1942), was about 15–20° C.

The results given by the two strains in the toxicity tests differed in degree, and are given separately. Mean log ED 50's are plotted against incubation temperature (Figs. 1 *a*, *b*, 2) or incubation time (Figs. 3–5). The vertical marks show the confidence limits in the mean log ED 50's. The limits (which varied in size around log ED 50 $\pm$ 0·02) were almost exactly the same size in parallel tests at different incubation temperatures, or in the same test after different incubation times.

Figs. 1 *a* and *b* show results with mercury compounds on strain A after incubation for 1 and 3 days at three temperatures. The temperature coefficients were mostly positive, increasing as incubation time passed, especially in the range 10–20° C. The 3-day 10:20° C. coefficients increased in the same order as the ED 50's at 10° C.; the sizes of these coefficients are shown on the lines in Fig. 1 *b*. In the range 10–25° C. the correspondence between toxicity and temperature coefficient was less exact. There

were two exceptions to the general pattern of positive coefficients. The 1-day 10:20° C. coefficient for mercuric thiocyanate and the 1-day 20:25° C. coefficient for phenyl mercury acetate were negative (Fig. 1*a*); the conidia were significantly more resistant to mercuric thiocyanate and less to phenyl mercury acetate at 20° C., after 1 day, than at the other temperatures. However, these negative coefficients did not persist when the conidia were kept longer; the 3-day temperature coefficients were positive for all mercury compounds over the whole temperature range (Fig. 1*b*).

Not surprisingly, there is less order among the results of the tests (on strain A) of the unrelated compounds shown in Fig. 2. The temperature coefficients, and the changes in ED 50 between the first and third days, were small. The largest 3-day 10:25° C.

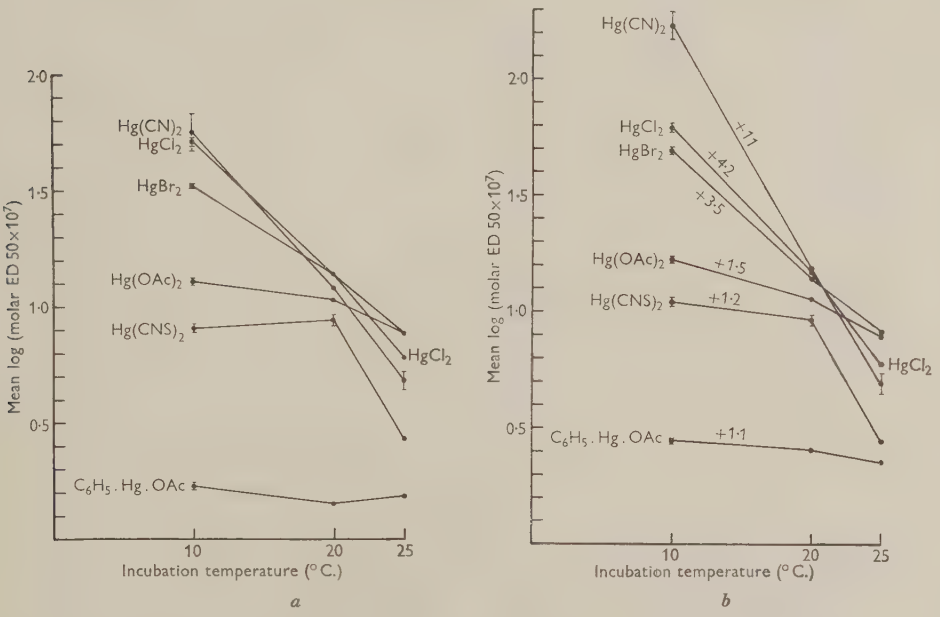

Fig. 1*a*. Results of tests of mercury compounds on conidia of strain A after incubation for 1 day (cf. Fig. 1*b*).

Fig. 1*b*. Results of tests of mercury compounds on conidia of strain A after incubation for 3 days (cf. Fig. 1*a*). The figures on the lines are the 10:20° C. temperature coefficients (ratios of ED 50's).

coefficient was given by *t*-butyl alcohol (+2·6), and the smallest by silver nitrate (+1·05). The conidia were significantly more resistant to copper sulphate and pentachlorophenol at 20° C. than at the other temperatures; but, in contrast to the tests with mercuric thiocyanate, the effects persisted until the third day.

After about 5 years of continuous culturing, growth of strain A in our cultures became progressively poorer, and it eventually became useless as a test organism. The size of the temperature coefficients also decreased at about the same time as the growth

in culture became poorer; thus, the 1-day 10:25° C. temperature coefficient of mercuric chloride was only +1·8, compared with +8·5 in the earlier tests (Fig. 1 *a*). Other strains of *B. fabae*, even though they grew vigorously in culture, also gave small 1-day temperature coefficients. Fig. 3 shows results of tests of mercuric cyanide on strain B, compared with the results on the original strain A (replotted from Figs. 1 *a*, *b*). The 1- and 3-day 10:25° C. coefficients were +12 and +35 (strain A), and +1·3 and +5·1 (strain B). In other tests on strain B, the 1-day 10:25° C. coefficients were, for mercuric chloride, +1·7; and for *t*-butyl alcohol, −1·5; the corresponding figures from the original strain A were +8·5 and +2·2.

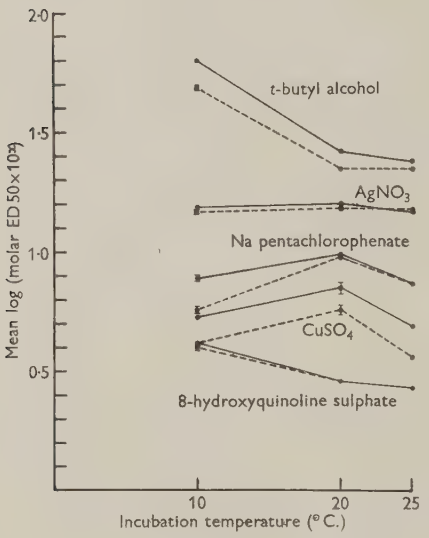

Fig. 2

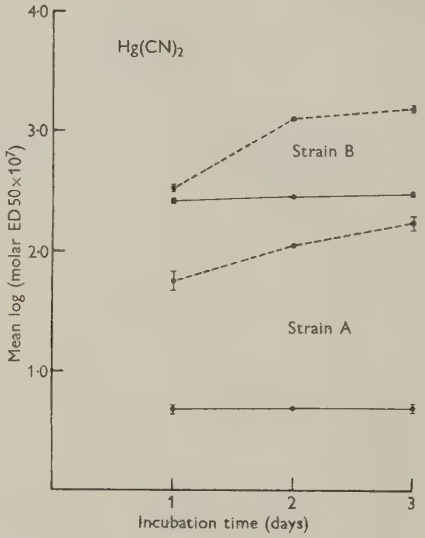

Fig. 3

Fig. 2. Results of tests on conidia of strain A after incubation for one day (broken lines) and 3 days (full lines). On the vertical scale, $x = 2$ for *t*-butyl alcohol, 5 for sodium pentachlorophenate and copper sulphate, and 7 for silver nitrate and 8-hydroxyquinoline sulphate.

Fig. 3. Results of tests of mercuric cyanide on conidia of strains A and B after incubation at 10° C. (broken lines) and 25° C. (full lines).

The results with mercuric cyanide illustrate the general tendency for the positive coefficients to increase with increase in incubation time; Fig. 3 shows this more clearly than Figs. 1 *a* and *b*.

Figs. 4 and 5 show results, plotted in the same way as in Fig. 3, with some organic compounds on strain B at 10 and 25° C. in distilled water (*t*-butyl alcohol) or in 0·05 M phthalate buffer (pyridine, 3-picoline, phenol, *p*-chlorophenol). In all these tests, as in those on strain A, the ED 50's increased with increase in incubation time, and the observed increases were greater at 10 than 25° C.; in addition, the ED 50's reached steady values after about 3 days at 25° C., or approached steady values after about

10–14 days at 10° C. The temperature coefficients in these tests on strain B were negative after 1 day, but became positive after 2–7 days. The 1-day and last-day 10:25° C. coefficients were, for *t*-butyl alcohol, −1·5, +3·2; for pyridine, −1·5, +2·4; for 3-picoline, −3·9, +1·7; for phenol, −1·7, +2·6; for *p*-chlorophenol, −1·7, +1·3.

In tests of some other organic compounds on strain B at 10 and 25° C., the 1-day coefficients were small and positive (about +1·7); the temperature coefficients and the ED 50's changed only slightly in size with increase in incubation time. These compounds, with the mean values for log (molar ED 50 × 10^7) after 1 day at 25° C. were: acetamide in distilled water, 6·50; *p*-nitrophenol at pH 4·0, 3·08; 2,4-dinitrophenol at pH 4·0, 1·63; methyl mercury nitrile in distilled water, 0·57.

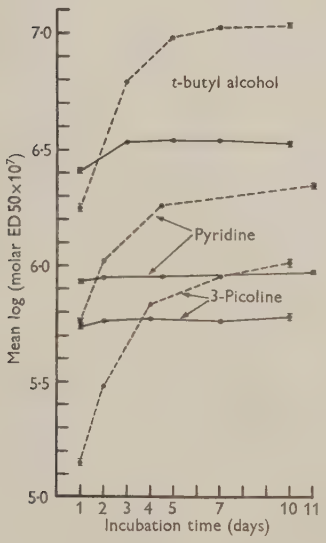

Fig. 4

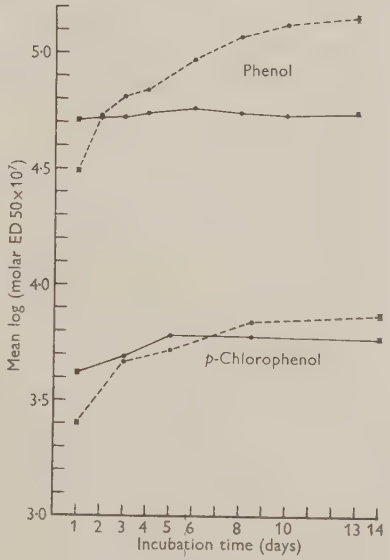

Fig. 5

Fig. 4. Results of tests on conidia of strain B after incubation at 10° C. (broken lines) and 25° C. (full lines).

Fig. 5. Results of tests on conidia of strain B after incubation at 10° C. (broken lines) and 25° C. (full lines).

DISCUSSION

(1) Although most of the effects of temperature on toxic action were small, they were consistent, and it is obvious that, in tests on one species, the relative toxicity of two fungicides may depend on the incubation time and temperature (cf. Tomkins, 1932; Suter, 1941), and on the strain of conidia used.

(2) In the tests on strain B (Figs. 3–5), the log ED 50's increased rapidly during the first few days of incubation, but the rates of change became progressively smaller as incubation time passed. Thus, the log ED 50–time lines were concave to the time axis

and convex to the log ED 50 axis. The effect of temperature on the curvature of these lines is not clear, because the changes in ED 50 at 25° C. were very small.

Wellman & McCallan (1942) found, in tests of the fungistatic action of five compounds on spores of three species at 21° C., that the log ED 50–time lines had this same general shape; and they obtained straight lines when log ED 50 was plotted against reciprocal of incubation time (but not against its logarithm). However, in my tests, the curvature of the lines (at 10° C.) did not follow any set pattern. Straight lines could be obtained by replotting mean log ED 50's against reciprocal of incubation time (pyridine) or against its logarithm (phenol); and the results with *t*-butyl alcohol, 3-picoline, and *p*-chlorophenol did not give straight lines by either transformation. The fact that some of the data happened to give straight lines, when transformed by one method or the other, seems to have been quite accidental (cf. Henderson Smith, 1921). If, as seems likely (McCallan & Wilcoxon, 1933), the ED 50's do in fact reach final steady values in this kind of test, such straight-line relationships can hold only during the earlier stages of the test. Wellman & McCallan's tests did not last for more than 4 days.

(3) In some tests for insecticides of diverse chemical types, Das & McIntosh (1961) found that, when insects were given single doses and the post-treatment time and temperature were varied, the ED 50's usually decreased as time passed, finally reaching steady values at the 'end-points'; and the observed changes in ED 50 were greater at the lower temperature. An increase in post-treatment temperature usually increased the speed of action of any one compound (i.e. decreased the time taken for the end-point to be reached); it also increased the curvature of the ED 50–time lines, and decreased the ultimate toxicity. The temperature coefficients were either negative, or changed from positive to negative as time passed.

My tests of fungicides, which were also of diverse chemical types, measured their fungistatic action; the conidia germinated in the presence of the compounds. This type of test corresponds in some ways to insecticide tests in which the temperature of 'continuous treatment' is varied (Pradhan & Mundkur, 1957); but if conidia of *B. fabae* take up the available fungicide from solution quickly and efficiently (McCallan & Miller, 1958), so that the conidia get what amounts to single doses, the two series of tests may in fact correspond more closely. However, in spite of any differences in method, there are some general similarities between the two sets of results.

In tests of fungistatic action, ED 50's cannot decrease as time passes; they may increase or remain constant (Wellman & McCallan, 1942). In my tests they increased, but approached steady values (10° C.) or reached steady values (25° C.); the observed changes in ED 50 were greater at the lower temperature used. An increase in incubation temperature increased the speed of action of any one fungicide (cf. Clark, 1901; McClellan, 1942) but, in contrast to the results with insecticides, increased the ultimate toxicity. Thus, the temperature coefficients changed in the opposite direction from the insecticide coefficients: they were either positive, usually increasing in size, or they changed from negative to positive as incubation time passed. Because of these continuous changes, a single sign and figure are seldom enough to define the temperature coefficient of a fungicide, or the relative toxicity of two fungicides.

(4) There was very little evidence for the accepted view (Anon., 1943) that spores

are more resistant to fungicides at their optimum temperature for germination (15–20° C. for conidia of *B. fabae*). In the tests where three temperatures were used (Figs. 1 *a*, *b*, 2), only three of the eleven compounds were less toxic at 20° C. than at 10 and 25° C. Thus, mercuric thiocyanate, pentachlorophenol and copper sulphate had negative 1-day 10:20° C. coefficients, but the peaks in the log ED 50–temperature lines disappeared (mercuric thiocyanate) or became smaller (pentachlorophenol) when the incubation time was increased to 3 days. This change was paralleled by the simultaneous increase in the percentage germination of untreated conidia at low incubation temperatures (p. 436).* Pentachlorophenol gave a negative 1-day coefficient in the range 10–25° C., as well as 10–20° C.; in this way it resembles *t*-butyl alcohol, etc. (Figs. 4, 5), which may also be less toxic at 20° C. than at 10 and 25° C. after short incubation times.

(5) The positive temperature coefficient of mercuric chloride at least was not caused by differences in sorption of fungicide on glassware, etc.; in the range 10–25° C., sorption increases very slightly with temperature (McIntosh, 1961). The same is no doubt true of the other heavy-metal salts.

(6) The results with different compounds showed similarities in general form, but there was no simple relationship between their chemical and physical properties and their temperature coefficients. This is not surprising. No such rule holds for similar tests with insecticides (Das & McIntosh, 1961), and the fungicides used here were possibly more diverse. They differed in their ionization as well as in their other physical and chemical properties, and in their visible effects on the growth of germ tubes (e.g. increase in number of tubes, distortion or branching of tubes, etc.). However, there are some regularities among the results given by the mercury compounds, some of which had unusually large temperature coefficients.

In groups of non-electrolytes of the same molecular weight, the sizes of the positive temperature coefficients of toxicity are sometimes inversely related to the rates of penetration of cell membranes, provided the membranes behave like homogeneous lipoid layers, which the non-electrolytes penetrate by diffusion. Rates of penetration often increase with oil:water partition coefficients, and with decrease in molecular weight. Thus, positive temperature coefficients of non-electrolytes of equal molecular weight are sometimes inversely related to their oil:water partition coefficients (Davson & Danielli, 1952). Solvent:water partition coefficients are often taken as guides to oil:water partition coefficients.

Some of the simple mercuric salts are exceptional, as salts, in that they are quite soluble in water, but are scarcely ionized in solution. Thus mercuric acetate is about 70% ionized, but the other simple salts tested on strain A are very poorly ionized. Their molecular weights lie within 20% of the mean; Table 2 lists their 3-day 10:25° C. temperature coefficients, percentages ionized and some of their solvent:water partition coefficients. The partition coefficients of the closely related mercuric cyanide, chloride and bromide fall in the same order for each of the four solvents shown; presumably the oil:water partition coefficients would also fall in this order. The

* Blumer & Kundert (1960, *Landw. Jb. Schweiz* (New Series), **9**, 465) found that in spore germination tests of three poisons on four species in the range 7–36° C., the spores were most resistant at the optimum temperatures for germination. However, the incubation time was only 16 hr.

(positive) temperature coefficients fall in the order to be expected from the partition coefficients; they increase with decrease in solvent:water partition coefficient, i.e. in the presumed order of decreasing rates of penetration.

The correlation seems to extend to phenyl mercury acetate, which is about 5% ionized and had a small temperature coefficient (+1·2) and large partition coefficients (e.g. for benzene:water, ~3·9; for chloroform:water, 24). However, rather surprisingly, it does not extend to the almost un-ionized mercuric thiocyanate, whose partition coefficients do not fit into the regular pattern given by the other salts.

Table 2. *3-day 10:25° C. temperature coefficients (strain A) and physical properties of some mercuric salts (see pp. 441–442)*

Mercuric salt	Temp. coeff.	Approx. ionization (%)	Approx. solvent:water partition coeff. for			
			C_6H_6	CCl_4	$CHCl_3$	$(C_2H_5)_2O$
Cyanide	+35	0·2	>0·00023	0·00014	>0·00045	0·023
Chloride	+10·5	1·0	0·087	0·00045	0·022	2·3
Bromide	+ 6·0	1·0	1·1	0·0079	0·31	4·7
Thiocyanate	+ 4·0	1·0	0·0032	>0·022	>0·046	>0·020

Thus mercuric cyanide, chloride and bromide, which have roughly the same high molecular weight and which, in contrast to other salts, exist almost entirely in the form of neutral molecules in aqueous solution, also resembled non-electrolytes in their temperature coefficients. They behaved as if their toxic action depended on penetration of cell membranes by diffusion, a step which is apparently slow and temperature-dependent. Their relative rates of penetration seem to be correlated with their partition coefficients and not, as with many ionizing compounds, with the percentages of neutral molecules in solution (Albert, 1960). On the basis of ionization alone, the least ionized salt (cyanide) would be expected to penetrate most readily and to have the smallest temperature coefficient; similarly, a highly ionized salt like the acetate would be expected to have a larger coefficient. Yet the acetate can evidently enter spores rapidly. Its temperature coefficient was small; and Owens & Miller (1957) showed that most of the mercury, from sublethal applied concentrations, had reached the cytoplasm within 1 hr.

(In this connexion, the fact that the temperature coefficients of mercuric cyanide, mercuric chloride and *t*-butyl alcohol were smaller on strain B than on strain A (p. 438 and Fig. 3) may be taken to show that the cell membranes were more permeable in strain B than in strain A.)

There seems little point in trying to relate the temperature coefficients of all five phenols tested to their physical properties. The last-day 10:25° C. temperature coefficients all lay in the narrow range +2·6 (phenol on strain B) to +1·05 (pentachlorophenol on strain A), but their physical properties varied enormously. Thus the molecular weights, benzene:water and ether:water partition coefficients, and percentages ionized of these two compounds only, are, for phenol, 94, 2·3, 44 and 0·0001%; and, for pentachlorophenol, 266, 14,000, 96,000 and 90%. To be toxic, phenols must in general penetrate cell membranes as neutral molecules; their toxicities increase with decrease in pH (Albert, 1960). At constant pH, the rates of penetration will depend on

the ionization as well as on the oil : water partition coefficients. The temperature coefficients of phenol and pentachlorophenol are in the order to be expected from their partition coefficients; but the difference in the rates of penetration (and in temperature coefficients) to be expected from the difference in partition coefficients is no doubt offset by the lower molecular weight and percentage ionization of phenol. Each of these properties would favour a relatively high rate of penetration.

As the correlation between temperature coefficients and physical properties within groups of related compounds is by no means clear, it is not surprising that there is no correlation when compounds of different chemical types are compared. Thus, three of the four compounds which gave identical small and unchanging temperature coefficients on strain B (p. 439) were 0·06 % ionized or less, but otherwise had a wide range of physical properties. Their molecular weights, benzene : water, chloroform : water and ether : water partition coefficients are, for acetamide, 59, 0·0017, 0·01 and 0·0025; for *p*-nitrophenol, 139, 1·2, 5·6 and 110; for methyl mercury nitrile, 242, 3·6, 2·5 and 0·38. Again, phenol and pyridine, whose molecular weights are approximately the same (94 and 79), differ widely in their physical properties. The ether : water and olive oil : water partition coefficients are larger for phenol than pyridine (44 and 6·0 compared with 1·2 and 1·0), but the reverse is true of the chloroform : water coefficients (3·5 compared with > 11); and phenol was very poorly ionized (0·0001 %), but pyridine well ionized (20–55 %). In spite of these differences, the last-day 10 : 25° C. temperature coefficients on strain B (+2·6 and +2·4), and the rates of change of temperature coefficient, were almost identical (Figs. 4, 5).

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REFERENCES

Albert, A. (1960). *Selective toxicity*, 2nd edn. London: Methuen.

Anon. (1943). The slide-germination method of evaluating protectant fungicides. *Phytopathology*, **33**, 627.

Anon. (1947). Test tube dilution technique for use with the slide-germination method of evaluating protectant fungicides. *Phytopathology*, **37**, 354.

Blackman, G. E., Parke, M. H. & Garton, G. (1955). The physiological activity of substituted phenols. II. Relationships between physical properties and physiological activity. *Arch. Biochem. Biophys.* **54**, 55.

Carswell, T. S. & Hatfield, I. (1939). Pentachlorophenol for wood preservation. *Industr. Engng Chem.* (*Industr.*), **31**, 1431.

Clark, J. F. (1901). On the toxic value of mercuric chloride and its double salts. *J. phys. Chem.* **5**, 289.

Collander, R. (1949). Die Verteilung organischer Verbindungen zwischen Äther und Wasser. *Acta chem. scand.* **3**, 717.

Das, M. & McIntosh, A. H. (1961). Effect of time and temperature on toxicity of insecticides to insects. III. Tests of seven poisons in the range 10–28° C. *Ann. appl. Biol.* **49**, 267.

Davson, H. & Danielli, J. F. (1952). *The permeability of natural membranes*, 2nd edn. Cambridge University Press.

Ephraim, F. (1948). *Inorganic chemistry*, 5th edn., revised by P. C. L. Thorne & E. R. Roberts. London: Gurney and Jackson.

FINNEY, D. J. (1952*a*). *Statistical method in biological assay*. London: Griffin.
FINNEY, D. J. (1952*b*). *Probit analysis*, 2nd edn. Cambridge University Press.
HEILBRON, SIR I. & BUNBURY, H. M. (1953). *Dictionary of organic compounds*, 3rd edn. London: Eyre and Spottiswoode.
HENDERSON SMITH, J. (1921). The killing of *Botrytis* spores by phenol. *Ann. appl. Biol.* **8**, 27.
HORSFALL, J. G. (1956). *Principles of fungicidal action*. Waltham, Mass: Chronica Botanica Co.
JENSEN, H. L. (1946). Observations on properties of certain fungicidal compounds. *Proc. Linn. Soc. N.S.W.* **71**, 119.
KNUDSEN, L. F. & CURTIS, J. M. (1947). The use of the angular transformation in biological assays. *J. Amer. statist. Ass.* **42**, 282.
LAST, F. T. & HAMLEY, R. E. (1956). A local-lesion technique for measuring the infectivity of conidia of *Botrytis fabae* Sardiña. *Ann. appl. Biol.* **44**, 410.
LONG, E. G. & KOBE, K. A. (1951). Hydrolysis of mercuric acetate. *Industr. Engng Chem.* (*Industr.*), **43**, 2366.
MCCALLAN, S. E. A. & MILLER, L. P. (1958). Innate toxicity of fungicides. *Advanc. Pest Control Res.* **2**, 107.
MCCALLAN, S. E. A. & WILCOXON, F. (1933). The form of the toxicity surface for copper sulphate and for sulphur, in relation to conidia of *Sclerotinia americana*. *Contr. Boyce Thompson Inst.* **5**, 173.
MCCLELLAN, W. D. (1942). Temperature as it affects spore germination in the presence of copper and sulphur. *Phytopathology*, **32**, 394.
MCINTOSH, A. H. (1961). Some variants and possible errors in the test-tube dilution and slide-germination methods for laboratory testing of fungicides. *Ann. appl. Biol.* **49**, 424.
MACY, R. (1948). Partition coefficients of fifty compounds between olive oil and water at 20° C. *J. industr. Hyg.* **30**, 140.
MARCUS, Y. (1957). Mercury (II) halide mixed complexes in solution. I. The experimental method and the distribution of the neutral complex. *Acta chem. scand.* **11**, 329.
MILLER, L. C. & TAINTER, M. L. (1944). Estimation of the ED 50 and its error by means of logarithmic-probit graph paper. *Proc. Soc. exp. Biol., N.Y.*, **57**, 261.
MILLER, V. L. & POLLEY, D. (1954). Determination of diphenylmercury alone or in presence of phenylmercuric compounds. Application to ethyl analogs. *Analyt. Chem.* **26**, 1333.
OWENS, R. G. & MILLER, L. P. (1957). Intracellular distribution of metal ions and organic fungicides in fungus spores. *Contr. Boyce Thompson Inst.* **19**, 177.
PRADHAN, S. & MUNDKUR, S. S. (1957). The effect of temperature on the susceptibility of mosquito (*Culex fatigans*) larvae to DDT suspensions. *Z. angew. Ent.* **40**, 371.
REITZ, O. (1939). Zur Säure- und Basenkatalyse in leichtem und schwerem Wasser. II. Hydrolyse und Deuteriumaustausch von Acetamid und Acetonitril. *Z. phys. Chem.* A, **183**, 371.
SANDELL, K. B. (1958). Über die Verteilung organischer Verbindungen zwischen Wasser und organischen Lösungsmitteln. *Mh. Chem.* **89**, 36.
SIDGWICK, N. V. (1950). *The chemical elements and their compounds*. Oxford: Clarendon Press.
SPEYERS, C. L. (1902). Solubilities of some carbon compounds and densities of their solutions. *Amer. J. Sci.* (4th series), **14**, 293.
SUTER, C. M. (1941). Relationships between the structures and bactericidal properties of phenols. *Chem. Rev.* **28**, 269.
TOMKINS, R. G. (1932). Measuring germination. *Trans. Brit. mycol. Soc.* **17**, 147.
WELLMAN, R. H. & MCCALLAN, S. E. A. (1942). An analysis of factors causing variation in spore germination tests of fungicides. IV. Time and temperature. *Contr. Boyce Thompson Inst.* **12**, 431.
YOUNG, S. & FORTEY, E. C. (1902). The properties of mixtures of the lower alcohols with water. *J. chem. Soc.* **81**, 717.

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Strains of *Botrytis allii* resistant to chlorinated nitrobenzenes

By D. PRIEST and R. K. S. WOOD
Botany Department, Imperial College, London, S.W. 7

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SUMMARY

When *Botrytis allii* was grown in nutrient agar in the presence of vapour of pentachloronitrobenzene (PCNB), or isomers of tetrachloronitrobenzene (TCNB), a slow-growing mycelium of distorted hyphae was produced on which sporulation was much reduced or suppressed. After periods which differed with the different fungicides, variants were produced which in the absence of fungicide closely resembled the parent but which, in the presence of fungicide, grew much more rapidly than the parent, and produced hyphae of normal appearance. A strain produced in response to one fungicide was more resistant than the parent to each of the other three fungicides, but the extent of this resistance depended on the origin of the strain. The most resistant was the 2,3,4,6-TCNB-resistant strain, followed in turn by the 2,3,5,6-TCNB-, 2,3,4,5-TCNB-, and PCNB-resistant strains.

In liquid culture, apart from the fact that fungistatic effects were less pronounced, the results were similar to those obtained on agar; the resistant strains utilized carbohydrate more efficiently than did the parent strain in the presence of each of the fungicides.

The strains resistant to PCNB and TCNB were also resistant, in varying degrees, to diiodo-, dibromo- and dichloronitrobenzenes. The diiodo- and dibromo- compounds were less active against *B. allii* than was TCNB, whereas the dichloro- compounds were more active and in certain conditions killed inocula. Resistant strains were also resistant to benzene, to 2,3,5,6-tetrachloronitroaniline, and to 2,6-dichloro-4-nitroaniline, the active principle of 'Allisan'. Resistant strains retained their resistance for at least 18 months under ordinary conditions of laboratory subculturing, and were as pathogenic to onions as was the parent strain.

INTRODUCTION

The chlorinated nitrobenzenes are unusual fungicides in at least two respects. They differ from most other fungicides in being active against some groups of fungi, but almost without effect against others even at very high concentrations, and in inducing the formation of resistant strains of certain susceptible fungi. These were first reported by Roy (1947), and Reavill (1950) for *Botrytis cinerea*, and have since been obtained by a number of other workers, notably Hewlett (1955), Brook & Chesters (1957), and Parry (1957); McKee (1951) obtained strains of *Fusarium caeruleum* which were also resistant. Such strains are different from those obtained in response to most other

types of fungicides in the following ways: (*a*) Generally, they appear 'spontaneously' in cultures growing slowly in high concentrations of the fungicide, and not after a period of 'training' in which the fungi are grown in increasing, but sublethal, concentrations of the fungicide; (*b*) although resistant strains sporulate less, or not at all, in the presence of the fungicide, vegetative growth may be almost unaffected, so that in this respect the strains are highly resistant; much lower levels of resistance are described for strains resistant to other types of fungicide; (*c*) resistant strains retain their resistance after long periods of growth in the absence of the fungicide; under similar conditions other types of resistant strains generally lose their resistance relatively quickly.

Despite the fact that resistant strains are readily obtained, and that chlorinated nitrobenzenes, in one form or another, are used in plant protection on an increasing scale, the problem and significance of resistance has so far received little attention, except in the work of Brook & Chesters (1957), and in work done intermittently with *Botrytis cinerea* for some time past at Imperial College. This paper summarizes work done more recently with *B. allii*; free use has been made of the techniques used, and the results obtained, by Hewlett (1955) and Parry (1957) with *B. cinerea*.

MATERIALS AND METHODS

Fungicides. Pentachloronitrobenzene (PCNB), and the 2,3,5,6-, 2,3,4,5-, 2,3,4,6-isomers of tetrachloronitrobenzene (TCNB) were kindly supplied by Mr A. F. Hams, Boots Pure Drug Co., Ltd. After recrystallization from acetone they had melting points of 146, 65–65·5, 39·5–41° C. Other halogenated nitrobenzenes, 2,5-dichloronitrobenzene (DCNB) (M.P. 54° C.), 2,4-DCNB (M.P. 33° C.), 2,3-DCNB (M.P. 61° C.), 2,5-dibromonitrobenzene (M.P. 84° C.), and 2,5-diiodonitrobenzene (M.P. 109° C.), were supplied by L. Light and Co., Ltd. They were recrystallized from ethyl alcohol (DCNB), or acetone (others) solutions before use. The active principle of the fungicide 'Allisan', 2,6-dichloro-4-nitroaniline, was supplied by Boots Pure Drug Co., Ltd. After recrystallization from acetic acid it had a M.P. of 194–195° C.

2,3,5,6-tetrachloroaniline was prepared by reduction of 2,3,5,6-TCNB; recrystallized from petroleum ether, it had a M.P. of 109–110° C.

Fungi and cultures. The strain of *B. allii* employed was isolated originally from an onion bulb. Although grown in culture for many years, it was still pathogenic to onion bulbs and caused quite typical rots in 7–14 days at 18–20° C. Cultures were grown on the following medium ± 2 % agar: glucose—1 %, peptone—0·2 %, KH_2PO_4—0·1 %, $MgSO_4.7H_2O$—0·05 % $FeSO_4.7H_2O$—0·001 %.

Agar plates and liquid cultures were generally inoculated with disks (5 mm. diam.) taken from the edge of non-sporing colonies which had grown for 72 hr. at 15° C. The disks were placed on fresh agar with the mycelium side downwards.

Spores needed for inoculation or germination tests were washed from the surface of agar slopes (7–14 days at 20° C.) with sterile water, passed through several layers of muslin, washed by centrifuging several times, and suspended in sterile water to a final concentration of 25,000/ml.

Spores were germinated at 20–22° C. in films on the surface of disks (10 mm. diam.)

of 3 % agar with 2 % sucrose kept in a humid chamber. They were considered to be germinated when they had produced a germ-tube equal to, or greater than, half the length of the spore; each treatment was replicated nine times.

Growth on agar plates was measured along two fixed diameters at right angles in each of ten replicates.

Mycelia from liquid cultures were collected on muslin for dry-weight determinations, washed with cold water, and dried for 24 hr. at 70° C.

EXPERIMENTAL

Effect of PCNB and TCNB on growth and sporulation of Botrytis allii

1 ml. acetone solution of known concentration of the fungicides was spread over the inside flat surface of lids of 9 cm. Petri dishes and allowed to evaporate in a sterile inoculating chamber. A more even film was obtained if the lids were cooled at about 3° C. beforehand. 24 hr. later inverted plates of agar which had been inoculated with the fungi a few minutes previously were placed above the films of fungicide, and incubated at 20–22° C. Each treatment contained ten plates, isolated in a metal box with a close-fitting, but not air-tight, lid. Vegetative growth was measured at intervals and the results are summarized in Table 1.

Table 1. *Effect of PCNB and TCNB on growth of* Botrytis allii *on agar plates*

	Mean increase colony diameter (mm./day)				Lag phase (hr.)*		
Concentration...	Nil	0·1 mg.	1 mg.	10 mg.	0·1 mg.	1 mg.	10 mg.
PCNB	15·9	—	6·8	6·2	—	<24	<24
2,3,4,5-TCNB	16·5	7·7	2·2	1·3	<24	96	96
2,3,5,6-TCNB	16·9	4·3	0·7	0·6	72	96	96
2,3,4,6-TCNB	16·9	1·0	0·7	0·6	48	96	96

* Time for appearance of growth from inoculum.

The general pattern of response of the fungus to each of the fungicides was similar. The early growth from inocula was of distorted, deeply pigmented cells, which formed nodular masses above the agar surface. Growth below the surface of the agar was much less irregular, and so was the later growth on and above the agar surface. The period when the fungus was confined to the inoculum (lag phase), was less than 24 hr. with PCNB at each dose, and with 0·1 mg. of 2,3,4,5-TCNB. With higher doses of 2,3,4,5-TCNB, and with each dose of the other isomers of TCNB the lag phase was much longer, e.g. with 10 mg. of 2,3,4,6-TCNB, although some growth from the inoculum could be detected *microscopically* after 96 hr., this had not extended more than 1 mm. in the next 170 hr. At the highest concentrations used, PCNB reduced the rate of linear growth by about 60 %; 2,3,4,5-TCNB by about 92 %, and the other two isomers by about 96 %. At the lowest concentration (0·1 mg.), 2,3,4,6-TCNB was considerably more effective than the 2,3,5,6-isomer, so that these four fungicides were effective in the order 2,3,4,6- > 2,3,5,6- > 2,3,4,5-TCNB > PCNB.

A somewhat unexpected feature of these results, particularly for 2,3,4,5- and 2,3,5,6-TCNB, was that different amounts of the fungicides had such different effects on the growth rate of the fungus because even at the lowest dosage there was far more fungicide than was required to saturate the air space above the lid. In fact, when the above experiment was finished after 30 days, the same lids were used in a second experiment, and it was found that lids with 10 or 1 mg. produced the same results as before, and that the lids with 0·1 mg. still reduced the rate of growth very considerably, although not to the same extent as previously.

Mycelium produced in the presence of PCNB sporulated freely; sporulation was considerably delayed and reduced by 2,3,4,5-TCNB and completely suppressed by 2,3,5,6- and 2,3,4,6-TCNB.

Resistant saltants appeared in some plates of all treatments except those with 0·1 mg. fungicide, and with 1 mg. PCNB. Time of appearance and number of plates producing them are given in Table 2.

Table 2. *Appearance of resistant saltants of* Botrytis allii

	Time (days) for first appearance		No. of plates with saltants*	
Concentration...	1 mg.	10 mg.	1 mg.	10 mg.
PCNB	Nil	5	Nil	3
2,3,4,5-TCNB	10	10	5	4
2,3,5,6-TCNB	13	20	4	6
2,3,4,6-TCNB	15	23	7	4

* Total possible—10.

The mycelium of these saltants was at first white but gradually became a pale creamy brown as it extended. Subcultures were prepared from these sectors and were used in later experiments.

In another series of experiments colonies of 2, 3, 4 or 5 cm. diameter were used in place of new inocula, and only one concentration of fungicide was used, 10 mg. per plate. In general, similar results were obtained but in detail they differed in the following ways. There was no lag phase with PCNB and 2,3,4,5-TCNB, the lag phase was no more than 24 hr. for 2,3,5,6-TCNB, and 72 hr. for 2,3,4,6-TCNB. No clearly defined saltants appeared in the PCNB plates, but saltants appeared in the 2,3,4,5-, 2,3,5,6-, and 2,3,4,6-TCNB plates after (approx.) 120, 168 and 288 hr., i.e. considerably earlier than when the inocula were used. Saltants in 2,3,4,5-TCNB plates, 8 days after introducing the fungicide, show a regular edge, contrasting with the irregular wavy outline of the slower-growing parent colony.

Germination of spores in presence of PCNB or TCNB. Suspensions of *B. allii* spores were spread in a thin film over the surface of agar disks containing 2 % sucrose placed in Petri dishes above 10 mg. of the fungicides distributed over the lids. Each treatment was replicated nine times and the results shown in Table 3 are based on the behaviour of some 200–400 spores of each replicate. PCNB had virtually no effect on germination and subsequent growth of germ-tubes; as in the controls, germination was complete after 12 hr. and a rudimentary mycelium had been formed after 24 hr.

The 2,3,4,5 isomer of TCNB retarded germination, and 2,3,4,6-TCNB was still more effective in this respect, but in time all the spores germinated. The 2,3,5,6 isomer was not much more active than PCNB. This was surprising in view of its effects on linear growth, but similar results were obtained when the experiment was repeated twice with different lots of spores.

Table 3. *Germination of* Botrytis allii *spores in presence of PCNB or TCNB*

		% germination			
			TCNB		
Incubation period (hr.)	Control	PCNB	2,3,4,5	2,3,5,6	2,3,4,6
4	96	88	2	56	0
8	98	95	12	92	0
12	100	100	56	100	7
16	100	100	67	100	24
24	—	—	94	—	47
72	—	—	100	—	95
118	—	—	100	—	100

Growth of resistant saltants in absence of fungicide. Isolates from five saltants which had appeared independently in plates exposed to each of the fungicides were first grown for 5 days in the absence of fungicides to reduce or eliminate any effects which might have been caused by the transfer of fungicide absorbed by the inocula. Disks were then transferred to fresh plates and the rate of growth was measured for 5 days. There was very little difference in the growth rates of different saltants produced in response to a particular fungicide, or between the growth rates of PCNB, 2,3,4,5- and 2,3,5,6-TCNB saltants (16, 17, 17 mm./day), but the growth rate of the 2,3,4,6-TCNB saltant was appreciably less (12 mm./day); the parent grew 16–17 mm./day.

Each of the colonies was similar in general appearance, form of hyphae, and sporulation, to colonies of the parent strain under the same conditions.

Growth of resistant saltants in presence of fungicides. In this series of experiments the parent and the four types of resistant strains were grown in the presence of 10 mg. of each of the four fungicides in turn. The data for the first 5 days' growth are summarized in Table 4, and show that resistance originating in response to one of the fungicides conferred some resistance to each of the others. This suggests that each of the four fungicides acts upon *B. allii* in some similar way, and that the fungus responds by producing the same adaptive mechanism. But additional mechanisms seem to be required to cope with TCNB and, more specifically, with the 2,3,4,6 isomer. Thus, each of the TCNB-resistant strains grew better than the PCNB-resistant strain in the presence of TCNB and the 2,3,4,6-TCNB-resistant strain was more resistant to 2,3,4,6-TCNB than were the other resistant strains.

In the second group of experiments, inocula were taken from the parent colony and from saltants arising from it. These were then grown in the presence of 10 mg. of each fungicide; the results are given in Table 5. There was no clear evidence that exposure of *B. allii* to any of the fungicides increased its resistance to PCNB, or that exposure to PCNB increased resistance to any of the other fungicides, but exposure

to TCNB did give some increase in resistance to each of the isomers. However, this increase was small compared with that of saltant mycelium from the same plate.

Growth of parent and resistant strains in liquid culture in presence of PCNB or TCNB. 1 ml. acetone solution containing 10 mg. fungicide was added to 0·5 ml. water in a sterile 250 ml. Erlenmeyer flask; the fine precipitate formed was distributed over the base of the flask as the liquid evaporated at room temperature. 50 ml. sterile glucose-peptone medium was added, the flasks were seeded with a disk (0·5 mm. diam.) taken from the edge of colonies of the parent or resistant strains growing on glucose-peptone

Table 4. *Growth of parent and resistant strains in presence of PCNB and TCNB*

Mean increase colony diameter (mm./day).*

	Strain			
		TCNB		
Fungicide	Parent	PCNB	2,3,4,5	2,3,5,6	2,3,4,6
Nil	17	16	17	17	17
PCNB	4·6	8·4	11·4	8·8	12·2
2,3,4,5-TCNB	1·0	1·6	4·6	4·4	4·4
2,3,5,6-TCNB	1·0	2·6	6·0	8·4	9·2
2,3,4,6-TCNB	1·0	1·2	2·6	2·2	4·0

* Mean for first 5 days' growth.

Table 5. *Growth of parent and saltant mycelium from the same plate in presence of PCNB and TCNB*

Mean increase colony diameter (mm./day).

	Fungicide				
			TCNB		
Pre-treatment	Nil	PCNB	2,3,4,5	2,3,5,6	2,3,4,6
None	15	6	1·1	0·6	0·5
PCNB: Parent	14	7·8	0·8	0·6	0·6
Saltant	13	8·2	2·2	5·3	0·8
TCNB-2,3,4,5: Parent	13	5·6	1·8	1·7	0·8
Saltant	15	9·4	4·3	8·4	2·6
2,3,5,6: Parent	14	4·7	1·2	2·0	0·6
Saltant	14	7·5	2·6	9·3	1·7
2,3,4,6: Parent	9	8·2	1·6	1·4	0·9
Saltant	12	10·4	3·4	8·0	4·2

agar, and incubated at 20–22° C. on a shaking machine. At intervals, two flasks of each treatment were removed and the dry weights of mycelia were determined. Table 6 gives the dry weight of mycelium per culture in each treatment of a series after a period of incubation in which the most rapidly growing member of the series had attained its maximum weight. In the absence of fungicide the parent and resistant

strains behaved similarly in all readily observable ways, and had produced very similar amounts of mycelium after 7 days when growth was at a maximum. Reducing-group determinations showed that by this time at least 90% of the glucose originally present had disappeared from the culture media.

In the presence of PCNB, the parent strain grew more slowly than each of the four resistant strains, and the differences were quite pronounced with samples taken 5 and 7 days after inoculation. But because the parent strain continued to grow after the resistant strains had attained their maximum weight, these differences got less in the later samples so that after 15 days the dry weights of mycelia of the parent and resistant strains were about the same. Apart from differences in the times to attain maximum dry weight the cultures of parent and resistant strains were very similar.

Table 6. *Growth of parent and resistant strains in liquid media*

		Strain				
				TCNB		
Treatment	Incubation (days)	Parent	PCNB	2,3,4,5	2,3,5,6	2,3,4,6
Nil	7	228	227	240	248	242
PCNB	7	127	207	183	178	247
2,3,4,5-TCNB	9	53	84	152	134	124
2,3,5,6-TCNB	10	31	38	72	142	132
2,3,4,6-TCNB	15	2	1	54	97	114

Figures shown are dry wt. (mg.) per culture.

TCNB affected the growth of the parent strain much more than did PCNB and, of the three isomers, the 2,3,4,6 was by far the most effective. In the presence of 2,3,4,5-TCNB, which was the *least* effective of the three isomers, there was visible growth from inocula after 2 days and this was followed by a slow and regular increase during which the abnormally shaped hyphae of the early growth were replaced by hyphae of normal appearance. Some aerial mycelium was produced at the liquid/glass interface, and on this mycelium there appeared a number of short aerial hyphae with dilated ends from which irregularly shaped cells were budded. These cells were not readily removed from the parent structure and might have been abortive conidia. Apart from the fact that mycelium was produced about one and a half times as quickly, the PCNB strain behaved in much the same way as the parent strain. In contrast, the TCNB-resistant strains grew 2·5–3 times as rapidly as the parent, and the initial period of abnormal growth was absent.

Analysis of the culture filtrates for reducing substances showed that each of the resistant strains produced about twice as much mycelium (per unit weight glucose consumed) than the parent strain (on the assumption that the reducing groups left in the medium belonged to residual glucose.)

The 2,3,5,6 isomer of TCNB was more fungistatic than the 2,3,4,5 isomer, but the relative effects on the different strains were similar except for the 2,3,4,5 strain which grew very slowly and abnormally in the 4 days after inoculation, and subsequently grew about half as fast as the 2,3,5,6- and 2,3,4,6-resistant strains.

In the presence of 2,3,4,6-TCNB neither the parent nor the PCNB-resistant strain

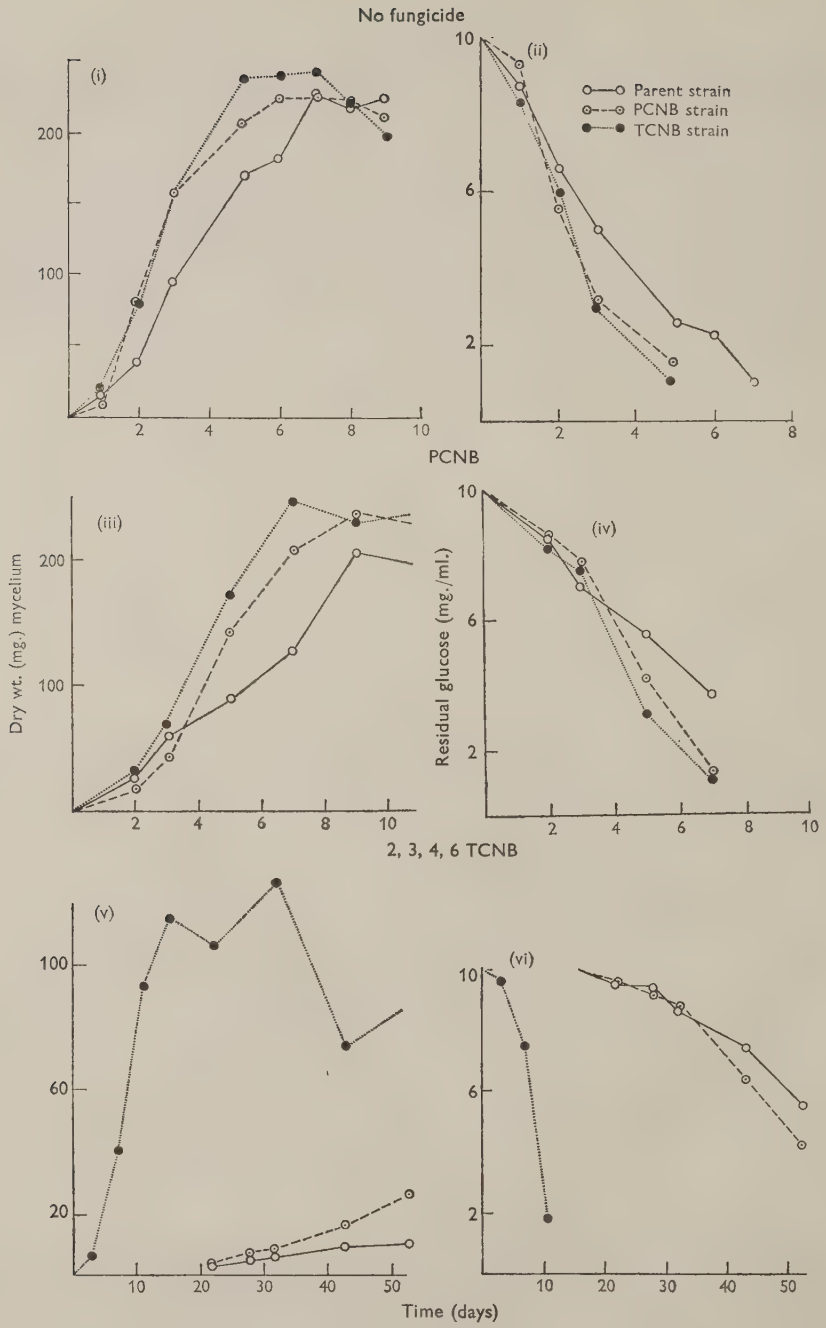

Fig. 1. Growth of parent, PCNB and 2,3,4,6-TCNB resistant strains of *Botrytis allii* in liquid media containing no fungicide, PCNB, or 2,3,4,6-TCNB.

grew visibly in the 15–18 days after inoculation, and grew only very slowly afterwards. After 28, 32, 43 and 53 days the parent strain produced no more than 4·6, 5·7, 8·2, and 11·2 mg. mycelium per culture, and the PCNB strain about 50% more in each case. In spite of the very poor growth at the end of 53 days about 50% of the original reducing groups in the medium had gone, giving low values of about 0·05 and 0·10 for the amount of mycelium produced per unit weight glucose consumed by the parent and PCNB strains respectively.

The three TCNB-resistant strains all produced hyphae of normal appearance 2–4 days after inoculation and, in their later growth, structures resembling the 'abortive' conidia described earlier. The 2,3,4,5 strain grew about half as well as the 2,3,5,6 strain which produced about 15% less mycelium than the 2,3,4,6 strain after 15 days' growth. The differences were more pronounced in samples taken earlier, e.g. after 11 days' growth the weights of the 2,3,4,5, 2,3,5,6 and 2,3,4,6 strain mycelia were respectively 25, 46 and 92 mg./culture. These figures show that the 2,3,4,6-resistant strain was the most resistant of the three.

Some of the results which have been summarized very briefly above are illustrated in Fig. 1 which shows the dry weight mycelia produced by the parent, PCNB- and 2,3,4,6-TCNB-resistant strains under different conditions, and the residual reducing groups in the liquid media. The different behaviour of the two resistant strains under the exacting conditions of exposure to the 2,3,4,6 isomer of TCNB is particularly striking.

Table 7. *Germination of spores of parent and resistant strains*

% germination after 24 hr.

	Strain				
			TCNB		
Treatment	Parent	PCNB	2,3,4,5	2,3,5,6	2,3,4,6
Nil	100	99	96	93	94
PCNB	100	99	95	94	95
2,3,4,5-TCNB	92	88	94	93	87
2,3,5,6-TCNB	99	98	97	92	94
2,3,4,6-TCNB	49	42	66	78	76

Germination of spores of parent and resistant strains. Spores were taken from 2-week-old cultures of parent and resistant strains grown on glucose-peptone agar in the absence of fungicides. Suspensions containing about 10^4 spores/ml. were spread over agar disks containing 2% sucrose suspended over 10 mg. films of the different fungicides in Petri dishes. Germination after 24 hr. at 22° C. is shown in Table 7. In the absence of fungicide the spores of the TCNB-resistant strains were slightly less viable than those of the parent strain. This result was obtained when the experiment was repeated a second time, and after incubation for another 24 hr. In the presence of PCNB, and 2,3,4,5- and 2,3,5,6-TCNB, there was not much difference in the behaviour of the parent and the four resistant strains, but there were differences when 2,3,4,6-TCNB was used. Germination of spores of the parent and PCNB-resistant strain was reduced by 50% or more whereas spores of the 2,3,4,6- and 2,3,5,6-TCNB-

resistant strains were much less affected. The 2,3,4,5-TCNB-resistant strain again behaved in an intermediate way.

Effect of other compounds on growth of parent and resistant strains. Dichloronitrobenzenes. Parent and resistant strains were grown on agar from standard disk inocula in the presence of 1 or 10 mg. of 2,3-, 2,4-, or 2,5-DCNB. Growth, as mean colony diameter, was recorded at suitable intervals and the results are summarized in Table 8.

With 10 mg. 2,5-DCNB none of the strains had grown after 93 days and when the lids of the Petri dishes were replaced by lids not carrying fungicide only the 2,3,4,6-resistant strain grew from the inoculum, and then only after a lapse of 7 days. Afterwards, it grew very slowly, at the rate of 0·5 mm./day, presumably because the agar had absorbed sufficient fungicide to prevent more rapid growth. The inocula of the other strains did not grow after transfer to fresh agar so it may be presumed that they were dead.

Table 8. *Growth of parent and resistant strains in presence of dichloronitrobenzene*

Increase colony diameter (mm./day).

		Strain			
			TCNB		
Treatment	Parent	PCNB	2,3,4,5	2,3,5,6	2,3,4,6
2,5-DCNB 1 mg.	2·4	1·6	3·1	5·2	7·0
2,5-DCNB 10 mg.	0	0	0	0	0
2,4-DCNB 1 mg.	0·4	0·7	1·2	4·3	8·4
2,4-DCNB 10 mg.	0	0	0	0	0·8
2,3-DCNB 1 mg.	0	0	0·1	0·01	0·2
2,3-DCNB 10 mg.	0	0	0	0	0·01

When 1 mg. 2,5-DCNB was used, the parent, PCNB- and 2,3,4,5-TCNB-resistant strains behaved rather similarly although they grew across the agar at somewhat different rates. The hyphae were at first abnormal in being yellow-brown in colour with parts of the walls wrinkled, and with conspicuous granulation of the cytoplasm; they were closely interwoven and formed nodular masses which tended to give the colonies an irregular shape. Later, after periods of 15–25 days for the different strains, hyphae of normal appearance were produced and these grew across the agar in a thin film. The colonies were sporulating lightly after 25–37 days.

In contrast, the 2,3,5,6- and 2,3,4,6-TCNB-resistant strains were almost unaffected by 2,5,-DCNB at this concentration. Regularly shaped colonies with hyphae of normal appearance were formed from the beginning, and there was abundant sporulation after 6–8 days. The only readily noticed difference between these cultures and those grown without fungicide was that in mass the immature conidia had a distinctly pinkish tinge.

At the higher concentration of 10 mg. there was a suggestion that 2,4-DCNB was less active than 2,5-DCNB because, although all inocula except those of the 2,3,4,6-TCNB-resistant strain were killed, this strain produced colonies 64 mm. diameter after 69 days, and these colonies sporulated lightly. But the use of lower concentra-

tions showed that 2,4-DCNB was, in fact, the more fungistatic of the two. The parent, PCNB- and 2,3,4,5-TCNB-resistant strains grew much more slowly in the presence of 2,4-DCNB and sporulation was delayed and reduced. All hyphae of colonies of the parent strain were abnormal and were similar to those produced at first by the two resistant strains. Later, however, the resistant strains produced normal hyphae which grew over the agar in a thin film. The 2,3,5,6-TCNB-resistant strain in a general way behaved similarly to the 2,3,4,5-TCNB-resistant strain, but in a number of respects it was much less affected. It grew more rapidly, produced normal hyphae more quickly, and sporulated sooner and more abundantly.

The 2,3,4,6-TCNB-resistant strain was yet again different because, apart from the fact that the hyphae had a yellowish tinge, colonies produced in the presence of 2,4-DCNB were similar in appearance and grew and sporulated almost as readily as did those grown without fungicide.

2,3-DCNB was a much more effective fungicide than the other two isomers. After exposure to 10 mg. only inocula of the 2,3,4,6-TCNB-resistant strain survived (17 out of 20 plates) and, even after the fungicide had been removed from the Petri dish, there was no growth from the inoculum for 47 days. Subsequently the fungus grew slowly over the surface as a creamy yellow colony of distorted hyphae which did not sporulate.

Even when only 1 mg. 2,3-DCNB was used, the inocula of the parent and the PCNB-resistant strain were killed. Inocula of the TCNB-resistant strains were not killed but the fungus grew from them only very slowly, and did not sporulate. The hyphae produced at first were abnormal in colour except in some colonies of the 2,3,4,6 TCNB-resistant strain where the hyphae were normal in appearance from the beginning. Later, similar hyphae were formed in the other colonies of this strain.

Dibromo- and diiodonitrobenzene. These were used in the same way as the DCNB compounds except that only the parent and the 2,3,4,6-TCNB-resistant strains were tested. With 10 mg. 2,5-DBNB, the parent strain grew at the rate of 0·4 mm./day and had not sporulated after 25 days' growth; the 2,3,4,6-TCNB-resistant strain grew 4·8 mm. daily, started to sporulate after 10 days, and was sporing freely after 25 days. When 1 mg. 2,5-DBNB was used the parent grew 1·0 mm. daily, and sporulated moderately after 20 days' growth. Growth and sporulation of the 2,3,4,6-TCNB strain was almost unaffected by this amount of fungicide.

2,5-DINB was considerably less fungistatic than 2,5-DBNB so that, at both concentrations, growth and sporulation of the 2,3,4,6-TCNB strain was hardly affected. At both concentrations the growth rate of the parent strain was about 30% that of the resistant strain; sporulation was delayed by 10–14 days and was not as heavy.

Against *B. allii*, therefore, these compounds were effective in the order DCNB > DBNB > DINB.

Tetrachloroaniline (*TCA*). The 2,3,5,6 isomer was prepared from 2,3,5,6-TCNB, 6 g. of which was mixed with 6 g. granulated tin and 42 ml. glacial acetic acid. The mixture was refluxed for 2 hr., diluted with water to 120 ml., made strongly alkaline with 50% NaOH, extracted with 40 ml. benzene which was dehydrated with anhydrous magnesium sulphate. After reducing the volume of solvent, 4·4 g. 2,3,5,6-tetrachloroaniline was crystallized, and purified by three successive crystallizations from light

petroleum ether. It was finally obtained as colourless, feathery crystals with a M.P. of 109–110° C. It was used in the ways described above at 10 mg./plate against the parent, the PCNB-, 2,3,4,5-, 2,3,5,6-, and 2,3,4,6-TCNB-resistant strains for which growth rates (mm./day) of 1·2, 2·3, 2·7, 10·0 and 9·2 were obtained. Growth and sporulation of the 2,3,5,6- and 2,3,4,6-TCNB-resistant strains were almost unaffected. Growth of the other three strains was greatly retarded by the fungicide and none had sporulated after 14 days. The parent strain grew at about half the rate of the other two, but after 8 days colonies of the parent strain produced well-defined sectors which grew rapidly across the plate. These saltants did not appear in the colonies of the PCNB- and 2,3,4,5-TCNB-resistant strain, but here the hyphae produced later were progressively less abnormal in appearance, and quite normal after 8–10 days.

2,6-*dichloro*-4-*nitroaniline*. This compound (DCNA) was introduced commercially a short time after the experiments with the tetrachloroaniline had been started. It is stated to be the active principle of the proprietary product 'Allisan' which is recommended for use against *Botrytis* spp. It was used against the parent and 2,3,4,6-TCNB-resistant strains at concentrations of 1 or 10 mg./plate. At both concentrations there was very little effect on the growth or sporulation of the resistant strain, and at 1 mg. per plate the parent strain was also not much affected: in each case colonies were sporulating freely after 4 days' growth. With 10 mg./plate, growth of the parent was reduced to about 2·5 mm./day and at first the hyphae were abnormal, but after 10 days, saltants appeared composed of hyphae of normal appearance which grew rapidly across the plate. Spores were produced somewhat sparsely on the parent colony after 6 days, but were formed abundantly on the mycelium of the saltants in the same plates. Subcultures from these saltants grew 6·5 mm. daily in the presence of 10 mg. fungicide, compared with 2·5 mm. for the parent, and 11·0 mm. for the 2,3,4,6-TCNB strain.

In glucose-peptone liquid media, the parent, DCNA- and 2,3,4,6-TCNB-resistant strains grew at much the same rate and appeared very similar in the absence of fungicide. In the presence of 10 mg. DCNA/50 ml. medium, growth rates of the three strains were 34, 120 and 211 mg. (dry wt.) during the first 7 days. The 2,3,4,6-TCNB-resistant strain was, therefore, more resistant than the DCNA-resistant strain, and, in fact, grew about as well in the presence as in the absence of the fungicide and in so doing produced an unusual effect, because the yellow crystals of the fungicide seemed to disappear from the culture after a few days. It was found later that they had become completely enmeshed by the growing mycelium so that the hyphae were, in effect, *growing around* the crystals.

Benzene. Cultures of the parent and 2,3,4,6-TCNB-resistant strains were placed in 17·5 l. desiccators to which were added sufficient benzene to give atmospheres containing approximately 1000, 2000, or 4000 p.p.m. benzene. At no concentration was there much effect on the growth and sporulation of the resistant strain. At 2000 and 4000, but not at 1000 p.p.m., benzene did retard the growth and sporulation of the parent strain but not to nearly the same extent as the substituted compounds used earlier. Thus, at 4000 p.p.m., the parent strain grew 4·8 mm. daily and was sporulating after 5 days. When the fungicide was removed the growth rate increased to that of untreated cultures, and the colonies spored abundantly. A characteristic of the new growth was

the appearance of numerous patches of fluffy, white aerial mycelium which remained sterile.

In the 9 days of the experiment, resistant saltants appeared only in cultures grown in 2000 p.p.m. benzene.

Pathogenicity of resistant strains. Disks, 5 mm. diameter, were taken from the edge of colonies of parent and each of the four resistant strains growing on glucose-peptone agar and put into holes, 5 mm. diameter, made in the tissue of healthy onion bulbs to a depth of the second or third fleshy scale. The plug of tissue was replaced, the wounds sealed with a Vaseline-wax mixture, and the bulbs incubated at 20–22° C. Typical *B. allii* lesions were produced by each of the strains, and there was no suggestion that the pathogenicity of the resistant strains differed from that of the parent strain.

DISCUSSION

Of the four compounds used in the main part of this work, only PCNB and 2,3,5,6-TCNB have been used to any extent commercially. From the results which have been obtained with PCNB against *B. allii in vitro*, and against other species of fungi by other workers (Reavill, 1950; McKee, 1951; Brook & Chesters, 1957), it is not easy to see why PCNB should give any substantial control of disease under field conditions. It has practically no effect on the germination of *B. allii* spores on a nutrient agar; on this agar and in liquid culture it does no more than reduce the rate of growth by about 50%, and it reduces sporulation, but not greatly. It might be objected that these laboratory tests were unnatural in the sense that the fungicide was applied to a fungus amply supplied with nutrients, and that under poorer nutrient conditions adverse effects on the fungus might be more pronounced and significant. This argument would have some force were it confined to the behaviour of spores on the intact surfaces of plants, but it is less sound if one considers that *Botrytis* spp. generally become established on dead or moribund tissues where nutrients are more abundant and more accessible. It is easier to understand why 2,3,5,6-TCNB should control diseases caused by *Botrytis* spp. because although it, too, does not prevent germination of *B. allii* spores, and only delays germination by a matter of hours, it does greatly retard growth of the fungus from inocula even on nutrient agar, and the colonies which are formed do not sporulate. Provided the fungicide *is* able to accumulate in host tissue to sufficiently high concentrations it can, therefore, be presumed that any lesions which are established will grow slowly, and that spread of the disease will be curtailed because spores will be produced very sparsely or not at all on the dead tissue.

In common with most other fungicides, 2,3,5,6-TCNB is insoluble so far as can be determined by ordinary chemical methods but is nevertheless fungistatic when suspended in water. The experiments with liquid media showed that under very favourable nutrient conditions, the fungus grew only very slowly with the fungicide at a concentration of 200 p.p.m. The effects of different dosages in liquid media were not determined but this was done in plate cultures, and it was found that most of the effect on linear growth was removed by reducing the dose per plate from 1 to 0·1 mg. Why there should be this effect at these concentrations is puzzling because in both cases the fungicide was deposited as a thin film on the lid of the Petri dish, and 0·1 mg. per

plate is much more than is required to saturate the volume of the plate; in fact at the end of the experiment there was sufficient fungicide left on the lids to reduce growth from new inocula on fresh agar. No explanation of these effects can be offered at the present time.

The 2,3,4,5 isomer of TCNB was intermediate in behaviour between PCNB and the 2,3,5,6 isomer, but 2,3,4,6-TCNB was, by a good margin, the most fungistatic of the four substances. The extent to which it retarded spore germination would be sufficient to account for its ability to control disease under field conditions (Brook & Chesters, 1957). Additionally, it all but prevented growth from inocula on agar plates, or in liquid media. It did not, however, kill the fungus. So far as the writers are aware, this isomer has not been used commercially to any extent, probably because it is difficult to produce on a large scale compared with 2,3,5,6-TCNB which approaches it in fungistatic properties.

The resistant strains produced in the presence of PCNB, and each of the three TCNB isomers were resistant in different degrees to each of the four substances. This suggests a mechanism of adaptation common to each of the four strains, and, therefore, that each of the four substances exert some of their effects on the fungus in a similar way. That there are additional mechanisms of toxicity and adaptation is suggested by the fact that although the PCNB-resistant strain does grow better than the parent strain in the presence of 2,3,4,6-TCNB, it is far more susceptible to this substance than is the 2,3,4,6-TCNB-resistant strain. Furthermore, and perhaps unexpectedly, the 2,3,4, 6-TCNB-resistant strain is more resistant to PCNB than is the PCNB-resistant strain. The behaviour of the other two resistant strains fitted the same pattern.

The dichloronitrobenzenes were far more effective against *B. allii in vitro* than was PCNB or any of the TCNB isomers. The different isomers of DCNB were increasingly active in the order 2,3 > 2,4, > 2,5 and their action differed from that of PCNB and TCNB in that inocula of parent and three of the four resistant strains were killed after exposure for long periods to 10 mg. fungicide per plate. The 2,3,4,6-TCNB-resistant strain survived this treatment. When lower concentrations, 1 mg. per plate, were used, the fungicidal and fungistatic effects of DCNB were less pronounced, and under these less exacting conditions it was found that resistance of the strains to DCNB increased in the order, 2,3,4,6- > 2,3,5,6- > 2,3,4,5-TCNB > PCNB. Hyphae more resistant than any of these four strains appeared in some of the cultures exposed to DCNB.

The bromine and iodine, 2,5-substituted nitrobenzenes were less active than the corresponding chlorine compound, and the 2,3,4,6-TCNB-resistant strain was almost unaffected by them. This strain and the 2,3,5,6-TCNB-resistant strain were also little affected by 2,3,5,6-tetrachloroaniline although the growth and sporulation of the parent and the other two resistant strains were retarded to about the same degree by this substance. The closely related 2,6-dichloro-4-nitroaniline was introduced fairly recently as the commercial fungicide 'Allisan', particularly against *Botrytis* spp. Although the growth of the parent strain was considerably reduced by this substance at the relatively high concentration of 10 mg./plate, there was much less effect at 1 mg./plate; resistant strains appeared frequently after 10 days and these grew rapidly and sporulated freely in the presence of the fungicide. Rather surprisingly, but in

agreement with other results, the 2,3,4,6-TCNB-resistant strain grew more rapidly than the DCNA-resistant strain in the presence of DCNA.

In considering the practical significance of these results it must be mentioned again that in view of the relatively poor performance of substances such as PCNB, TCNB and DCNA against *B. allii* in the laboratory it is surprising that they are found to be effective in the field. This problem is not confined to *Botrytis* spp.; PCNB is used on a fair scale against *Rhizoctonia solani*, but in culture it does not retard the growth of this fungus very much (de Silva & Wood, 1961). A possible explanation of these facts is that these substances, as well as acting upon the fungi, also modify the host so as to increase its resistance to disease. This is not improbable in view of the fact that in sufficiently high concentrations PCNB and TCNB profoundly alter the growth of some plants (Brown, 1947; Brown & Reavill, 1954). Lower concentrations which have no readily observed effect on growth might well alter the susceptibility of the tissue to infection. This has been demonstrated for other growth-regulating substances on tomatoes and *Fusarium oxysporum* f. *lycopersici* by Corden and Dimond (1959). If this happened when PCNB, TCNB or DCNA were applied to plants, it could also explain why any resistant strains which appeared might not have much effect on the incidence of disease because an adaptation which permitted the fungus to grow in the presence of the fungicides need not permit the fungus to overcome a form of resistance induced by the fungicide in the host.

It seems improbable that ordinary field conditions would present *B. allii* with suitable opportunities for developing resistant strains, because even in the laboratory these developed only after a lapse of some days, and in high concentrations of fungicide. But the results obtained in this work suggest that under other conditions where the fungus might be exposed for some time to relatively high concentrations of the fungicide, e.g. in frames, resistant strains could appear, and, if the results with *B. allii* are typical, these strains would retain the pathogenicity of the parent. Here it is also important to remember that the resistant strains produced in the laboratory retained their resistance for at least 18 months under ordinary conditions of subculture. As yet, there are no reports of resistance developing in the field but so far as the writers are aware, no systematic search has been made for them, and in this connexion it may be significant that in a recent paper Way & Keyworth (1959) have stated that, periodically, chlorinated nitrobenzenes fail to give satisfactory control of *B. cinerea* on lettuce. These are the circumstances in which it might be profitable to look for resistant strains of the pathogen.

REFERENCES

Brook, M. & Chesters, C. G. C. (1957). The growth of *Botrytis cinerea* Pers., *Fusarium caeruleum* (Lib.) Sacc. and *Phoma foveata* Foisters in the presence of tetrachloronitrobenzene isomers. *Ann. appl. Biol.* **45**, 498.

Brown, W. (1947). Experiments on the effect of chlorinated nitrobenzenes on the sprouting of potato tubers. *Ann. appl. Biol.* **34**, 422.

Brown, W. & Reavill, M. J. (1954). Effect of tetrachloronitrobenzene on the sprouting and cropping of potato tubers. *Ann. appl. Biol.* **41**, 435.

Corden, M. E. & Dimond, A. E. (1959). The effect of growth-regulating substances on disease resistance and plant growth. *Phytopathology*, **49**, 68.

Hewlett, M. A. (1955). Studies on the fungicidal activity of certain chlorinated nitrobenzene compounds. Ph.D. Thesis, University of London.

McKee, R. K. (1951). Mutations appearing in *Fusarium caeruleum* cultures treated with tetrachloronitrobenzene. *Nature, Lond.* **167**, 611.

Parry, K. (1957). A study of some factors affecting the susceptibility of fungi to fungicides. Ph.D. Thesis, University of London.

Reavill, M. J. (1950). The effect of certain chloronitrobenzenes on plant growth. Ph.D. Thesis, University of London.

Roy, R. Y. (1947). A comparison on the mode of action of certain new chloronitrobenzene preparations with that of standard fungicides. Ph.D. Thesis, University of London.

de Silva, L. & Wood, R. K. S. (1961). Unpublished results.

Way, J. M. & Keyworth, W. G. (1959). Experiments on the control of *Botrytis* disease of lettuce. *Ann. appl. Biol.* **47**, 685.

Ann. appl. Biol. (1961), **49**, 461–472
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Infection of bean plants (*Vicia faba* L.) with *Botrytis cinerea* and *B. fabae*

By B. J. DEVERALL and R. K. S. WOOD
Botany Department, Imperial College, London

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SUMMARY

The infection of bean plants (*Vicia faba* L.) by *Botrytis fabae* has been studied by inoculating detached leaves with standard volumes of spore suspensions of known concentrations, or with single spores.

Increasing the number of spores in the inoculation drops increased the number of lesions, and lesions were more readily produced on older than on younger leaves.

The addition of glucose, a number of other carbohydrates, and also sodium polypectate and pectin, increased the number and rate of the spread of lesions. Xylose caused a decrease, and a number of nitrogen compounds had little effect.

The number and rate of spread of lesions was increased if the surface of leaves were gently rubbed with a mild abrasive before inoculation. Calcium-deficient plants were more susceptible than plants which had received normal nutrition. Deficiencies of other major elements had much less effect.

A larger proportion of the spores in inoculation drops germinated when these drops were on older leaves, when the drops contained glucose, and on leaf surfaces that had been gently abraded. Addition of xylose reduced the amount of germination and the fungus grew very poorly in liquid culture when this was the only carbon source. The addition of small quantities of glucose allowed the fungus to use xylose as efficiently as glucose.

When single spores were used to inoculate leaves, very different results were obtained with leaves taken from different plants, but under some conditions single spores often induced lesion formation.

B. fabae caused lesions more readily than did *B. cinerea*, in spite of the fact that a greater proportion of *B. cinerea* spores in inoculation drops germinated.

INTRODUCTION

'Chocolate spot' is the name given to a disease of beans (*Vicia faba* L.) which appears on plants in the field as discrete, dispersed brown lesions, mainly on the leaves. This first, 'non-aggressive' stage, in which the lesions remain limited in size, occurs annually in Britain, but when weather conditions are suitable, particularly in May, June and July (Wilson, 1937; Hogg, 1956), it is succeeded by a second 'aggressive' stage in which infected tissue is almost black, and the lesions cause extensive leaf necrosis so that the plant itself may be killed in a relatively short period. When conditions are sufficiently humid the blackened tissue becomes covered with spores;

this rarely happens in the 'non-aggressive' phase. Wilson (1937) was one of the first to study this disease intensively and he concluded that in Britain the most important causal agent was *Botrytis cinerea* Pers., but stated that isolates of *B. fabae* Sard. from Spain and Japan caused a 'similar type of infection'. *B. fabae* Sard. was first recognized as a species by Sardiña (1929) who recorded it on beans in Spain where it caused a disease similar to Chocolate spot. Ogilvie & Munro (1947) showed that in south-west England Chocolate spot was caused mainly by *B. fabae*, and on the basis of field observations and laboratory tests Leach (1955) also concluded that, in Britain, *B. fabae* is responsible for the aggressive and, therefore, damaging phase of the disease.

The object of the present work was to compare the infection of bean plants by these two fungi, and to see how the size and nutrition of inocula, and the condition of the host, influenced infection by *B. fabae*.

MATERIALS AND METHODS

(*a*) *Bean plants.* In most of the experiments plants were grown from seeds of one sample of a commercial field bean variety 'Gartons' S.Q. Giant Winter Bean'. They were grown to the four-leaf stage, normally reached 21–27 days after sowing, in a greenhouse where the temperature seldom went below 15° C. except during cold spells. During winter months additional illumination of approximately 100 f.c. was provided for 12 hr. each day by 'Warm White' fluorescent tubes. Seed were sown in sand in Plain Waxed Containers with screw-top Plain Wax Lids, size 240, supplied by Mono-Containers Ltd. Drainage and stem holes were cut in the bases and lids and the cut surfaces were rewaxed. Washed sand came from Double Arches Pit No. 21 (Messrs Arnold, Quarries) as recommended by Hewitt (1952) and was used as obtained. Plants were watered three times weekly with the nutrient solution described by Hewitt (1952) and characterized as 2/1 in terms of mg. equiv. Ca''/K' per litre.

(*b*) *Fungi. B. cinerea* and *B. fabae* were isolated from lesions on diseased bean plants taken from a Berkshire field in June 1958; cultures were finally obtained from single spores.

Both fungi were grown on Medium 'X' described by Last & Hamley (1956), modified to include 3% agar; 20 ml. were put as a shallow layer in 150 ml. 'Pyrex' or 'Hysil' Erlenmeyer flasks, and after inoculation flasks were incubated at 21–23° C. in light from 'Osram Natural' fluorescent tubes at an intensity of 150 f.c. Under these conditions both fungi quickly produced a dense carpet of sporulating mycelium; spores were harvested in water 10–15 days after inoculation, the suspension filtered through three layers of sterile muslin, which removed most of the hyphal fragments, and the spores washed by centrifuging three times in sterile water before finally suspending in an appropriate medium at a known density.

(*c*) *Inoculation of plants.* After the fourth leaf had fully opened, all leaves were washed in a stream of distilled water and allowed to dry before they were detached and placed on a layer of muslin over a frame of glass rods on filter paper moistened with sterile water in transparent polystyrene boxes ($20 \times 12 \times 4$ cm.) with closely fitting lids. Drops (0·0002 ml.) of spore suspensions were put on the upper surfaces of the leaves from a hypodermic needle attached to an 'Agla' micrometer syringe.

The concentrations of the suspensions were adjusted so that on the average drops contained 10, 100, or 500 spores. Spores of *B. fabae* are large (20 μ long) and settled out rapidly, so it was necessary to resuspend them at frequent intervals; this problem was far less serious with the smaller (12–15 μ long) spores of *B. cinerea*.

It was not possible to dispense single spores with certainty because to maintain an *average* of one spore per drop, it would be necessary for some drops to contain no spores or more than one spore. Instead, small volumes of spore suspensions were spread over the surface of 1·5 % agar which had been washed in water and pyridine as described by Robbins (1939). Areas with single spores were identified under a binocular microscope and disks (1 mm. wide, 0·5 mm. thick) bearing the spore were transferred to the leaf surface.

The bifoliate leaves of young bean plants can be regarded as two half-leaves divided by the mid-vein into two half-leaflets. Last & Hamley (1956) showed there was no significant difference between the development of lesions on the four half-leaflets of one leaf when these were inoculated in the same way. In view of this, most experiments were designed so that four treatments could be compared on single leaves, variation between plants and leaves being reduced by using between four and eight plants for each experiment. Ten drops were placed on each half-leaflet.

After inoculation, leaves were incubated at 15° C., and the numbers of inoculations producing lesions after 24–48 hr. were counted; later, the proportion of the inoculated surface, usually one half-leaflet, which had become blackened as a result of infections was estimated.

(*d*) *Spore germination.* Drops (0·0002 ml.) with a known number of spores were put on disks (1 cm. diam.) cut from leaves of plants at the four-leaf stage, and floated undersurface down on sterile water in Petri dishes at 15° C. Later, disks were transferred to fixative (50 % ethanol, propionic acid, 40 % HCHO—90,5,5, by volume) under vacuum for 24 hr., cleared in lactophenol, and stained in lactophenol containing 0·05 % cotton blue. Germination and the early stages of lesion formation were readily detected in this way.

EXPERIMENTAL

Factors affecting production of lesions by Botrytis fabae

(*a*) *Effects of leaf age and spore numbers.* These were studied in two experiments each with four plants; the results are given in Table 1.

In both experiments the youngest leaf was considerably more resistant than the most susceptible of the other three. In the first experiment the oldest leaf was much more susceptible than the other three and this was the usual result in most experiments; the results of the second experiment were unusual in that the second youngest leaf was relatively highly susceptible.

The effects of spore numbers in the inocula were similar in the two experiments. Increasing the average number of spores from 10 to 100 per drop about trebled the number of lesions, and from 100 to 500 about doubled the number. The increased infectivities of larger inocula were of the same order on the oldest and most susceptible, and on the youngest and most resistant leaves.

The germination of spores at the leaf surface was studied by taking a large number

of disks from washed leaves at positions 1 to 4 on plants, floating them on water, and inoculating each with five drops containing 10, 100 or 500 spores of *B. fabae*; 3, 6, or 22 hr. later three disks from each leaf no./spore concentration combination were removed, and fixed and stained in the way described earlier. The number of spores, and the proportion which had germinated, at each point of inoculation was then determined, and the results are shown in Table 2.

Table 1. *Effect of leaf age and spore numbers on production of lesions*

Experiment	Possible lesions per treatment	No. of lesions (%)* Leaf number 1 (oldest)	2	3	4 (youngest)
1	120	63	28	13	15
2	120	32	30	36	5
		Spores per drop 10	100	500	
1	160	12·5	37	66	
2	160	12·5	33	52	
1 and 2 combined	80 leaf 1	15	50	75	
	80 leaf 4	2·5	11	18	

* As % possible.

Table 2. *Effect of leaf age and spore numbers on spore germination*

Incubation (hr.)	Leaf	Spores found (%) Spores per drop 10	100	500	Germinated (% found) Spores per drop 10	100	500
3	1 (oldest)	25	25	17	51	21	21
	2	3	5	43	20	24	9
	3	4	13	8	17	6	9
	4 (youngest)	6	4	13	44	12	4
6	1	37	35	28	48	12	21
	2	7	8	28	18	12	8
	3	60	15	12	21	5	4
	4	27	31	19	17	2	2
22	1	100	95	37	75	28	29
	2	67	69	48	77	29	23
	3	45	60	28	48	6	27
	4	89	52	48	23	16	12

Although there were some exceptions, after fixing and staining, more spores were retained by the older leaves, and when inocula contained fewer spores. Of the spores which were retained on the leaf surface, a considerably higher proportion germinated when the inoculum drop had contained 10 as compared with 100 and 500 spores, for which similar figures were obtained. And, although there were again exceptions, a greater proportion of spores germinated on the older leaves.

Lesions on the leaf disks were counted after 22 hr. and the usual results were obtained in that there were more lesions on older leaves and with larger numbers of spores.

The condition of spores which were removed during fixation and staining is not known but if it be assumed that they were ungerminated (because if they had germinated they would have been retained by appressoria) the mean numbers of germinated spores at the points of inoculation are shown in Table 3.

Table 3. *Number of germinated spores at inoculation points*

Incubation (hr.)	Leaf	No. of spores germinated. Spores per drop 10	100	500
3	1 (oldest)	1·2	5	20
	2	0·07	1	20
	3	0·07	0·7	3·5
	4 (youngest)	0·3	0·5	3·0
6	1	1·8	4	30
	2	0·1	0·9	10
	3	1·2	0·7	2·5
	4	0·5	0·6	1·5
22	1	7·5	26	55
	2	5·1	20	55
	3	2·1	3	40
	4	2·0	8	30

Although the proportion of spores which germinated decreased as the spores became more concentrated in a drop, the actual numbers of germinated spores increased with the size of the inoculum. Numbers of germinated spores also increased with leaf age, and both trends paralleled increases in the numbers of lesions produced. Observations on the development of individual lesions showed that six were caused by the germination and penetration of very few of the spores which were present. On the same disks, and over larger lesions, 90% of the spores had germinated and produced extensive germ-tubes. Unless there were marked differences in spore germinations at positions less than 1 cm. apart on leaf surfaces, these results suggest that substances had come from the lesions and stimulated the germination of spores on the surface.

(*b*) *Production of lesions by single spores.* Four plants were used and five spores of *B. fabae* were spread equidistantly down the centre line (base–apex) of each half-leaflet. For the experiment as a whole, 10% of the spores produced lesions, but of the total number, 72% were on the first plant, 19% on the second, and 9% on the other two together. On the first, most susceptible plant, the oldest leaf was much more susceptible than the youngest, and the middle two were intermediate in this respect.

Examination of the leaves showed that *each* of the 320 spores had germinated, and that the general appearance of the germ-tube was similar from leaf to leaf. From these data, it appeared that whether or not a lesion developed at the point inoculated depended more on differences in the tissue of different leaves and plants, than on differences between individual spores.

(*c*) *Effect of carbohydrates added to inocula.* In each of six experiments there were four plants, and a total of 64 half-leaflets distributed among four main treatments,

which were spores suspended in water or 1 % solutions of three different carbohydrates, and four subsidiary treatments, 0, 10, 100, 500 spores per drop: each treatment was, therefore, applied to four half-leaflets. Representative results for glucose, xylose and lactose are given in Table 4.

With inocula containing only ten spores, glucose considerably stimulated the production of lesions. Similar effects with larger inocula would have been masked by the fact that almost the maximum possible number of lesions were produced when spores were suspended in water. But the presence of glucose in the inoculum drop increased the spread of the lesions at all levels of inoculation.

Xylose reduced the number of lesions particularly when inocula contained 100 spores, and also limited the spread of the lesions compared with the controls.

Fructose, mannose and sucrose increased the number and spread of lesions in much the same way as did glucose.

Arabinose, ribose, galactose, maltose and cellobiose also increased the number of lesions, but not to the same extent as glucose, and they had relatively little effect on subsequent spread compared with the controls.

Corresponding figures for certain carbohydrate polymers in 1 % solutions are shown in Table 5.

Table 4. *Effect of carbohydrates on production of lesions*

	No. of lesions (%). Spores per drop			Leaf area diseased (%). Spores per drop		
Carbohydrate	10	100	500	10	100	500
Glucose	99	97	100	15	47	60
None*	64	99	100	5	7·5	15
Xylose	14	16	70	0	0	5
None	21	80	94	2·5	7·5	15
Lactose	24	23	71	0	6	17
None	56	43	70	0	14	34

* Spores suspended in water.

Table 5. *Effect of carbohydrate polymers on lesion production*

	No. of lesions (%). Spores per drop			Leaf area diseased (%). Spores per drop		
Carbohydrate	10	100	500	10	100	500
None	14	57	88	2·5	17	20
Sodium polypectate	44	80	79	0	37	40
Pectin	75	86	69	5	32	45
Carboxymethyl cellulose (D.S. 0·7)	8	31	61	0	7·5	10

Sodium polypectate and pectin considerably increased the number and spread of lesions whereas carboxymethyl cellulose reduced both.

The effects of 1 % solutions of glucose and xylose on the germination of spores (100) in drops on leaf disks floating on water are shown in Table 6. Twice as many spores were retained when the spores were suspended in the glucose solution compared

with water or xylose solution, and xylose greatly reduced, and glucose greatly increased the germination of the spores which were retained. The effects of glucose and xylose on the formation of lesions may, therefore, be explained, at least in part, in terms of their effects on spore germination. Indeed, xylose reduced spore germination so much that it was surprising that any lesions were produced when it was used.

Table 6. *Effect of glucose and xylose on spore germination*

Medium	Spores found (%)	Spores germinated (as % found)	Total spores germinated per inoculation
Water	33	19	6·3
Glucose	74	52	35·8
Xylose	32	1	0·3

Table 7. *Effect of carbohydrates on growth*

Carbohydrate	Effect on production and spread of lesions	Dry wt. (mg.) per culture
Glucose	Very stimulatory	37·2
Mannose		39·9
Fructose		16·0
Ribose	Moderately stimulatory	7·1
Arabinose		11·1
Galactose		18·4
Xylose	Inhibitory	28·1

Besides altering the amount of germination, carbohydrates might influence the formation and spread of lesions by affecting the growth of germ-tubes after germination. This was investigated by growing the fungus on certain of the carbohydrates used in inoculation experiments. The basal medium contained 0·05 % glucose, 0·2 % peptone, 0·1 % KH_2PO_4, 0·05 % $MgSO_4.7H_2O$ and (as p.p.m.) Fe 0·2, Zn 0·2, Cu 0·02, Mn 0·02; sugars were added at a final concentration of 1 % and the pH of the solution was adjusted to 5·0 with 0·1 M NaOH before autoclaving; 50 ml. medium in 250 ml. Erlenmeyer flasks were inoculated with 1 ml. of a dense suspension of spores of *B. fabae* and incubated on a shaking machine at 25° C. for 7 days when the weights of the washed mycelia were determined after drying at 70° C. for 24 hr. in an oven with forced draught ventilation. The results are shown in Table 7.

Growth on glucose, mannose, ribose, arabinose and galactose were in line with the effects of these substances on formation and spread of lesions. Fructose supported less growth than would have been expected on this basis but a more important anomaly was the very good growth on xylose. The growth experiments differed from the others in that the xylose was accompanied by small quantities of glucose, so the interaction between these sugars was studied in the treatments shown in Table 8.

The basal medium was the same as that shown above but without the 0·05 % glucose. There was negligible growth on water, 1 % glucose and 1 % xylose, and growth on (basal medium + xylose) was no better than that on basal medium alone.

The addition of 0·05 % glucose doubled growth on the basal medium, and allowed the fungus to utilize xylose as efficiently as glucose.

(*d*) *Effect of nitrogen compounds added to the inoculum.* These were tested at a final concentration of 0·03 M nitrogen in water, or in 0·007 M phosphate buffer at pH 4·5. The compounds tested were $NaNO_3$, NH_4Cl, NH_4 tartrate, glycine, alanine, aspartic acid, glutamic acid, l-asparagine and arginine. The buffer had little effect on the results. Ammonium tartrate, aspartic and glutamic acids caused some increase in numbers and spread of lesions, but were not nearly so effective as glucose. With the other substances the results were much as in the controls.

(*e*) *Effect of abrading leaf surface.* Leaves, untreated or lightly dusted with washed diatomaceous filter-aid (Hyflo Super Cel), were rubbed gently with the forefinger, washed with water and then allowed to dry before they were inoculated in the usual way with spores (100 per drop) suspended in water. The development of lesions after 3 days is shown in Table 9.

Table 8. *Effect of glucose and xylose on growth of mycelium*

Treatment	Dry wt. (mg.) mycelium
Water	0
Basal medium	9·2
1 % glucose	0·1
1 % xylose	0·2
Basal medium + 0·05 % glucose	18·6
+ 1 % glucose	68·3
+ 1 % xylose	10·5
+ 0·05 % glucose + 1 % xylose	69·8

Table 9. *Effect of abrasives on production of lesions*

	No. of lesions (%)			
	Leaf no.			
Treatment	1	2	3	4
Control	60	30	0	0
Abrasive	95	90	100	100

Rubbing the leaves with abrasive increased the number of lesions which were formed on the older, more susceptible leaves, and even more striking results were obtained with the younger leaves, in which no visible lesions developed on the controls.

The corresponding effects on the behaviour of the spores was determined by taking disks from untreated leaves, and from leaves lightly dusted with abrasive after these had been gently rubbed with the forefinger and then washed, and inoculating them with spores suspended in water (Table 10).

Rubbing with the abrasive did not affect the retention of the spores by the leaves but about trebled the proportion of the spores that germinated, and the germ-tubes were longer and thicker than those on leaves not treated in this way. The increase in the number of lesions caused by abrasion is, therefore, probably explained in part by a direct effect on spore germination.

(*f*) *Effect of host nutrition.* In each of a series of experiments, the susceptibility of plants grown in a complete nutrient medium was compared with that of plants grown on this medium less calcium, magnesium, potassium, nitrate or phosphate. Each of these deficiencies affected the growth of the plants to a greater or lesser degree; calcium deficiency had a particularly striking effect. After leaves had been detached from plants at the four-leaf stage, inoculated and incubated in the usual manner, it was found that those from nitrate- and phosphate-deficient plants behaved as did those from control plants. The production and spread of lesions seemed to be greater on magnesium- and potassium-deficient leaves but statistical analysis showed that the differences were not significant. The only results significant in this way were those from calcium-deficient leaves and these are shown in Table 11.

Table 10. *Effect of abrasives on spore germination*

Treatment	Spores found (%)	Spores germinated (as % found)	Total germinated spores per inoculation
Untreated	52	10	5·4
Abrasive	49	34	16·6

Table 11. *Effect of calcium deficiency on susceptibility*

	No. of lesions (%). Spores per drop			Leaf area diseased (%). Spores per drop		
Treatment	10	100	500	10	100	500
Complete	12	25	52	5	7·5	12·5
Less calcium	24	52	70	14	25	39

More lesions were established on calcium-deficient plants at all levels of inoculation, and the proportion of the half-leaflets covered by lesions after 6 days was also much greater. Similar effects of calcium deficiency in altering susceptibility to disease have been reported by Khan (in Kernkamp *et al.* 1952) for *Rhizoctonia solani* and soybeans, and by Edgington & Walker (1958) for *Fusarium oxysporum* f. *lycopersici* and tomato plants.

Comparison of infectivity and germination of conidia of Botrytis fabae *and* B. cinerea

Development of lesions on plants. The results of three experiments with different lots of plants are shown in Table 12.

For the same amount of inoculum, in terms of spore numbers, *B. fabae* produced 1·9 to 4·1 times as many lesions as *B. cinerea.* Also, *B. fabae* lesions spread much more rapidly. Sporulation never occurred over the discrete lesions, but only after the general blackening of the leaf, so *B. fabae* produced many more spores. Under these conditions, therefore, *B. fabae* was much more pathogenic than *B. cinerea.*

For *B. fabae* spores it has been shown that increased production of lesions may in part depend on increased germination of spores at the leaf surface. It was of interest, therefore, to compare the germination of the two types of spore at the leaf surface.

This was done by inoculating leaf disks from leaves 2 and 4 with three drops each containing 100 spores. Germination after 19 hr. is shown in Table 13.

More spores of both fungi germinated on the older leaves and there were also more lesions on these leaves. *B. fabae* caused more lesions on both types of leaves, but a much larger proportion of *B. cinerea* spores germinated at the points of inoculation. This implies that the differences in pathogenicity between the fungi are not directly attributable to differences in germination at the leaf surface.

Table 12. *Lesion production by* Botrytis cinerea *and* B. fabae

Experiment	Fungus	Possible lesions per treatment	No. of lesions (%). Spores per drop		
			10	100	500
1	*B. cinerea*	80	25	20	40
	B. fabae	80	61	65	75
2	*B. cinerea*	80	24	23	31
	B. fabae	80	82	96	100
3	*B. cinerea*	240	—	19	20
	B. fabae	240	—	58	82

Table 13. *Germination of spores of* Botrytis cinerea *and* B. fabae

Fungus	Leaf no.	Spores found (%)	Spores germinated (as % found)	No. of lesions (as % possible)
B. cinerea	2	80	43	8
	4	47	20	0
B. fabae	2	88	19	33
	4	94	6	17

DISCUSSION

The main objects of this work were to compare the infection of bean leaves by *Botrytis fabae* and *B. cinerea*, and to see how infection was affected by a number of different factors. An attempt was made to reduce the variability in disease development from one experiment to another by standardizing the size of the inocula and the method of inoculation, and by growing the host plants in as uniform a way as was possible with the facilities available. Only partial success was obtained and various results suggest that variability in disease development was mainly caused by differences in susceptibility of the host tissues. Some of these, such as differences between leaves of different ages, can be overcome by designing the experiment in a suitable way, and it is possible that between-plant variability could be reduced by using inbred lines of selected varieties because it is thought that commercial stocks are very variable genetically. There remains, however, the variability attributable to lack of uniformity in the growing conditions for plants used in different experiments. It is unlikely that mineral nutrition was important in this connexion because it was reasonably well standardized, and because experiments showed that

the susceptibility of plants to infection was not greatly affected by nutrient deficiencies except of calcium.

Heat and light are left as the main factors which will need to be controlled more carefully if variability is to be reduced in this type of experiment.

Comparison of the effects of different factors on the development of lesions and on the behaviour of *B. fabae* spores on the leaf surface showed that more spores germinated and more lesions were formed on older leaves, in the presence of certain sugars, and after the leaf surface had been rubbed gently with a mild abrasive. Conversely, xylose reduced both spore germination and the development of lesions. When inoculation drops containing different numbers of spores were used it was found that although the proportion of spores germinating was lower in drops with many spores, and these were drops which gave most lesions, the *total* number of spores germinating was greater than in drops with fewer spores. So it seems that increased development of lesions is in some way associated with factors which increased spore germination. However, when single-spore inocula were used it was found that although each of the spores germinated, the number which produced a lesion varied greatly from tissue to tissue. The ability to germinate and grow at the leaf surface is, therefore, quite distinct from the ability to infect and cause a lesion. Along somewhat different lines, Last (1960) has shown that spores of *B. fabae* lose their ability to infect more quickly than they lose their ability to germinate. It is probable that each of the treatments that increased spore germination also increased the rate and type of growth at the leaf surface, and that this is important in determining whether or not a lesion is produced. In this connexion it may be pointed out that when the data of twelve experiments in which the results were in suitable form were analysed in the way used by Meynell & Stocker (1957), it was found that the slope of the curve, log numbers of spores $\times$ % lesions as probits, was in each case below 2. This can be taken as evidence that synergistic action between spores is not an important factor in lesion formation, although the variability of the response of leaves to infection might reduce the validity of this conclusion.

B. fabae causes lesions on detached leaves much more readily than does *B. cinerea*, although a considerably larger proportion of the *B. cinerea* spores germinate. At present it is not known whether these differences in pathogenicity depend on differences in ability to penetrate into the leaf, or to induce symptoms after penetration. Certain differences in the physiological behaviour of the two fungi which may be connected with these processes are described in a second paper.

The effect of xylose on spore germination and growth of *B. fabae*, with and without small traces of glucose, has a bearing on the different behaviour of spores at the surface of young and older leaves and which might be explained by the suppression of germination on young leaves, or the stimulation of germination on older leaves. If stimulatory substances were involved, then the fact that xylose prevented germination on leaves suggests that they are not sugars which could act like glucose to nullify the inhibition of germination by xylose. Brown (1922) also found that the substances which passed into drops of water on bean leaves to increase the conductivity, unlike those from other plants, did not stimulate germination of *B. cinerea* spores.

The leaves of calcium-deficient plants were more susceptible to infection by *B. fabae* than were leaves from more normal plants; it is suggested that this was because

the walls of calcium-deficient cells are more readily degraded and penetrated by the pathogen. Calcium is believed to play an important role in the structure of the cell wall because of its property of forming linkages between adjacent chains of pectic acid. The rigidity of the gel increases with the frequency of such linkages, and this is shown by the commercial 'hardening' of tomato fruit by soaking them in solutions of calcium salts. It is also possible that calcium-deficient cell walls are more permeable to substances which stimulate the germination and growth of the pathogen at the leaf surface; this was not investigated.

Little information was obtained on what makes a lesion 'aggressive', although it was found that generally factors which increased the *number* of lesions caused by *B. fabae* also increased the spread of these lesions. When spores were suspended in water, 'aggressive lesions' were most readily obtained with high concentrations of spores on older leaves. Although competition for nutrients prevented the germination of many of the spores at first, later observations suggested that after a few spores had penetrated the leaf and caused a small lesion to develop many of the remaining spores now germinated so that a large number of germ-tubes covered the lesion. Under these conditions, therefore, an aggressive lesion was not caused by an initial mass attack by the fungus, but by massive support of a small lesion arising from the activity of a few of the spores in the inoculum.

REFERENCES

Brown, W. (1922). Studies in the physiology of parasitism. VIII. On the exosmosis of nutrient substances into the infection drop. *Ann. Bot., Lond.* **36**, 101.

Edgington, L. V. & Walker, J. C. (1958). Influence of calcium and boron nutrition on development of *Fusarium* wilt of tomato. *Phytopathology*, **48**, 324.

Hewitt, E. J. (1952). Sand and water culture methods in the study of plant nutrition. *Commonwealth Agric. Bur. Tech. Comm.* no. 22.

Hogg, W. H. (1956). Weather and the incidence of chocolate spot of beans. *N.A.A.S. Quart. Rev.* **32**, 87.

Kernkamp, M. F., de Zeeuw, D. J., Chen, S. M., Ortega, B. C., Tsiang, C. T. & Khan, A. M. (1952). *Tech. Bull. Univ. Minn. agric. Exp. Sta.* no. 230.

Last, F. T. (1960). Longevity of conidia of *Botrytis fabae* Sardiña. *Trans. Brit. mycol. Soc.* **43**, 673.

Last, F. T. & Hamley, R. (1956). A local lesion technique for measuring the infectivity of conidia of *Botrytis fabae* Sardiña. *Ann. appl. Biol.* **44**, 410.

Leach, R. (1955). Recent observations on the *Botrytis* infection of beans. *Trans. Brit. mycol. Soc.* (*Abstr.*) **38**, 171.

Meynell, G. G. & Stocker, B. A. D. (1957). Some hypotheses on the aetiology of fatal infections in partially resistant hosts and their application to mice challenged with *Salmonella paratyphi*-B or *Salmonella typhimurium* by intraperitoneal infection. *J. gen. Microbiol.* **16**, 38.

Ogilvie, L. & Munro, M. D. (1947). Occurrence of *B. fabae* Sard. in England. *Nature, Lond.* **160**, 96.

Robbins, W. J. (1939). Growth substances in agar. *Amer. J. Bot.* **26**, 772.

Sardiña, J. R. (1929). Una nueva especie de *Botrytis* que ataca a las Habas. *Mem. R. Soc. Espanola Hist. Nat.* **15**, 291.

Wilson, A. R. (1937). The chocolate spot disease of beans (*Vicia faba* L.) caused by *Botrytis cinerea* Pers. *Ann. appl. Biol.* **24**, 258.

Ann. appl. Biol. (1961), **49**, 473–487
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Chocolate spot of beans (*Vicia faba* L.)—interactions between phenolase of host and pectic enzymes of the pathogen

By B. J. DEVERALL and R. K. S. WOOD
Botany Department, Imperial College, London

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SUMMARY

Botrytis fabae and *B. cinerea*, pathogens which cause 'chocolate spot' of beans (*Vicia faba* L.) produced polygalacturonase abundantly in media containing pectic substances or soluble cellulose derivatives; only filtrates from cultures grown on polypectate had pectinesterase activity and even this was relatively low. *B. cinerea* filtrates from media containing cellulose had appreciable cellulase (Cx) activity, but did not degrade insoluble wood cellulose; *B. fabae* filtrates were inactive in both respects. Cultures of both fungi on certain types of media readily macerated sections of bean stems and caused them to become brownish black in colour.

Filtrates from cultures of both fungi had considerably lower polygalacturonase activities when leaf extracts were present in otherwise suitable media.

The latent phenolase present in water extracts of leaves was activated by polypectate, polygalacturonic acid, pectin and carboxymethylcellulose. Activation was most rapid near pH 4·5 and was negligible at pH 5·5 which was the optimum value for sodium dioctyl sulphosuccinate, one of the best activators so far described. Solutions of the polymers could be diluted with no great effect on activations.

Lesions are produced more readily on old than on young leaves, and extracts of old leaves discolour more rapidly than do extracts of young leaves when exposed to activated phenolase from bean leaves. This is because young leaves contain some system which retards the development of the coloured products which are normally produced following the oxidation of phenols.

Water extracts of bean leaves inactivate the polygalacturonase of both fungi. The polygalacturonase of *B. fabae* differed from that of *B. cinerea* in being rapidly inactivated after filtrates had been diluted with water, and by being inactivated quickly by cyanide. *B. fabae* polygalacturonase was inactivated relatively quickly by products formed after oxidation of dihydroxyphenylalanine, but was less affected when catechol was used; *B. cinerea* behaved in the opposite way.

The mechanisms by which both fungi cause lesions on bean leaves are discussed in the light of the results summarized above.

INTRODUCTION

Under greenhouse and laboratory conditions, two species of *Botrytis*, *B. fabae* Sard., and *B. cinerea* Pers., cause a disease of beans, *Vicia faba* L. aptly termed 'chocolate spot', but in the field only *B. fabae* is important (Ogilvie & Munro, 1947; Leach, 1955).

The initial stages of the diseases caused by both fungi are similar, and they result in the formation on the leaf, more rarely on other parts of the plant, of a discrete lesion, chocolate brown in colour, and limited in size (generally 1–3 mm. in diameter). If the plants are growing well in conditions which are not particularly humid, the lesions remain small and although the fungus can be recovered from the diseased tissue, it does not sporulate on the surface. When conditions are particularly suitable for the pathogen, the lesions spread rapidly and the 'spot' effect is lost because the pathogen colonizes large areas of leaf tissue in a matter of a few days. The diseased tissue becomes black in colour and the fungus often sporulates profusely on the dead leaf surface. This later stage of the disease, for which the term chocolate spot is not really appropriate, has been called the 'aggressive phase' and is more commonly caused by *B. fabae* than by *B. cinerea*.

There is now good evidence that cell-wall degrading enzymes play an important part when lesions are formed in succulent tissue by bacteria or fungi, and it is reasonable to suppose, therefore, that these enzymes are active in the formation of chocolate spot lesions. Also, the lesion, at least in the later stages, has a colour similar to that of tissue damaged mechanically and here the colour is likely to be produced through the interaction of phenolases and phenols in damaged host cells. It was decided, therefore, to study some of the cell-wall degrading enzymes produced by these two pathogens, the phenolase system in leaves of the host, and possible interactions between the two systems, in the hope that the information obtained would shed some light on the mechanism of lesion formation, and, more particularly, on why the lesions are often so limited in size.

MATERIALS AND METHODS

Some of these have been described in an earlier paper (Deverall & Wood, 1961). Others were as follows.

(*a*) *Pectic substances*, obtained from Sunkist Growers Inc., California, were high methoxyl pectin, sodium polypectate and polygalacturonic acid. Each was washed in 60 % ethanol containing 0·1N-HCl, in 95 % ethanol until free of chloride by the silver nitrate test, in absolute alcohol, and then air-dried at laboratory temperature.

(*b*) *Cellulose*. Soluble carboxymethylcellulose (CMC) was supplied by the Hercules Powder Co. Two samples, each with a degree of substitution of 0·7 (DS 7), were used; one was of medium, the other of higher chain length.

The insoluble cellulose was 'Solka Floc', from the Brown Paper Co., Boston, U.S.A., a finely divided wood product stated to contain at least 99·5 % cellulose.

(*c*) *Sodium dioctyl sulphosuccinate* (NaDSS) was obtained as 'Manoxol OT' from Hardman and Holden Ltd., Manchester.

(*d*) Phenols, sugars and various salts, were *Analar* grade reagents from British Drug Houses, who also supplied 'Difco' vitamin-free casamino acids.

(*e*) *Buffers*. Unless otherwise stated these were mixtures of appropriate volumes of 0·1M citric acid and 0·2M-Na_2HPO_4 for the desired pH.

(*f*) *Enzyme solutions* were obtained from cultures of the fungi on media containing 0·05 % glucose and 1 % of different carbon sources, 0·46 %—casamino acids (Difco), 0·1 %—KH_2PO_4, 0·05 %—$MgSO_4.7H_2O$, and (as p.p.m.), 0·2—Fe, 0·2—Zn, 0·02—

Cu, 0·02—Mo, and 0·02—Mn. The pH of the media was adjusted to 5·0 before 50 ml. volumes were autoclaved in 250 ml. Erlenmeyer flasks and then inoculated with 1 ml. of a dense spore suspension. Cultures were incubated on a shaking machine at 25° C. for 7 days before mycelia were harvested by filtration and centrifuging, washed, dried at 70° C., and then weighed. Culture filtrates were made cell free by centrifuging, and frozen at −20° C. until required.

(*g*) *Leaf extracts* were prepared by freezing leaves quickly at −20° C., and grinding them while frozen in a volume of ice water (ml.) ten times the fresh weight in g. After centrifuging at 10,000 r.p.m. at 4° C. for 10 min., extracts were used immediately or frozen at −20° C. until required. Dialysed extracts were obtained by dialysing the ground tissue in approximately 100 volumes of a 10-fold dilution of the buffer for 18 hr. at 4° C.; the buffer was stirred magnetically during the dialysis. The extracts were then centrifuged before storage.

(*h*) *Pectic enzyme activities* were determined in the following ways:

(1) *Polygalacturonase* (PG) by measuring reduction of viscosity of solutions of pectic substances [PG (V)], or measuring increase in reducing groups [PG (R)]. Viscosity was determined with Cannon–Fenske viscometers at 25° C.; reaction mixtures contained 5 ml. 1 % solution of pectic substances, NaOH or HCl to bring to the required pH, 1 ml. 0·05 M sodium citrate buffer at an appropriate pH, 1 ml. test solution, and water to 10 ml. There was a linear relation between enzyme concentration and time required to cause a given viscosity change. Activity is expressed as $100/t$ (min.) to cause 50 % reduction in viscosity.

Reducing groups were measured in 5 ml. samples taken from mixtures at 25° C. containing 10 ml. 1·5 % solution of the pectic substance, NaOH or HCl to produce the required pH, 5 ml. buffer, 5 ml. test solution and water to 40 ml.; the Willstätter–Schudel hypoiodite method as modified by Jansen & MacDonnell (1945) was used for determining reducing groups, and activity is expressed as the percentage hydrolysis of available glycosidic linkages in unit time.

(2) *Pectinesterase* (PE) was estimated by measuring carboxyl groups liberated at 25° C. in mixtures containing 15 ml. 1 % pectin, 1 ml. 0·2 M-NaOH to bring the pH to 5·5, 5 ml. test solution and water to 25 ml. After the enzyme solution had been added the pH, continuously recorded by a pH meter, was maintained near the initial value by the addition of 0·02 M-NaOH. Activity is expressed as the percentage hydrolysis of methyl ester linkages in unit time.

(*i*) *Cellulase activity* was estimated in the ways described for polygalacturonase except that the reaction mixtures contained carboxymethyl cellulose or wood cellulose in place of the pectic substances.

(*j*) *Phenolase activity* was determined colorimetrically with mixtures containing 3·5 mg. catechol or dihydroxyphenylalanine (DOPA), 1 ml. buffer, 1 ml. activated enzyme solution, and water to a final volume of 7 ml. After adding the enzyme, extinction values were read in an 'EEL' colorimeter with filter 623, at intervals over 200 sec. Extinction changes over the period 20–200 sec., plotted against enzyme concentration, gave a linear relationship.

(*k*) *Activation of phenolase.* The phenolase present in leaf extracts was activated by a number of substances in the following way; 0·2 ml. dialysed leaf extracts was added

to a mixture of 0·6 ml. water, 0·14 ml. activator solution and 0·06 ml. buffer. This was allowed to stand at 18–20° C. for 15 min. and phenolase activity was then determined. A standard activation was given by NaDSS at a final concentration of 4 mM.

EXPERIMENTAL

I. *Pectic and cellulolytic enzymes produced in culture*

(*a*) *Pectinesterase* activities of filtrates from cultures grown on pectin were lower than those from polypectate cultures. This was unexpected because secretion of PE is generally induced by its substrate, but it is possible that the ezyme *was* secreted in the pectin cultures and inactivated later by their low pH which was finally in the region of 3·5. The activity of the polypectate filtrates and, for comparison, water and salt extracts (0·2 M-NaCl at pH 7·0) of fresh bean leaves, is shown in Table 1 where the figures represent the percentage hydrolysis in the first 5 min. of the reaction at pH 5·5. The *B. cinerea* filtrates were much more active than were those of *B. fabae*, but they were relatively inactive compared with the salt extracts of leaves. The difference between the water and salt extracts of the leaves is one generally obtained with tissues of higher plants. Salt and water extracts of the mycelia of the two fungi from these cultures were inactive.

Table 1. *Pectinesterase activity of culture filtrates and leaf extracts*

Culture filtrates		Leaf extracts*	
B. fabae	*B. cinerea*	Water	Salt
0·1	0·5	0·7	19·0

* 0·5 g. fresh wt. leaves in 25 ml. 1·0 % pectin solution.

Table 2. *PG (V) activity and growth of cultures*

Fungus	Carbon source	Dry wt. (mg.) mycelium	PG (V) on polypectate
B. fabae	Glucose	23	2
	Pectin	18	20
	Polypectate	36	50
B. cinerea	Glucose	253	1
	Pectin	74	6
	Polypectate	152	50

No further tests were made with *B. fabae* filtrates because of their low activity, but activity of *B. cinerea* filtrates at pH 4·0, 4·7, 5·5 and 6·3 was found to be 0·6, 0·7, 0·5 and 0·05.

(*b*) *Polygalacturonase activity*. The growth of cultures, and the PG (V) activity at pH 5·5 of filtrates from cultures on different carbon sources is shown in Table 2.

On each of the substrates *B. cinerea* grew far more than *B. fabae*, but there were no corresponding differences in enzyme secretion in this experiment, and, in fact, *B. fabae* filtrates were as active as or more active than the *B. cinerea* filtrates. This, however,

was not the usual result because if in a series of polypectate culture filtrates obtained for different purposes the activity of the *B. fabae* filtrate is expressed as 100, the activities of the *B. cinerea* filtrates were: 50, 125, 178, 250, 125, 222, 312, 233 and 200; so that, generally, *B. cinerea* filtrates were two to three times as active as *B. fabae* filtrates in degrading polypectate. Table 2 also shows that active filtrates were obtained only when pectic substances were present in the culture media; this is the result which has been obtained with many other fungi.

The pH optimum for PG (V) of both fungi was about 6·0 for degradation of polypectate, and somewhat lower, 5·0–5·5, for pectin. In contrast the PG (R) activity on pectin and on polypectate was higher at pH 4·5 than at pH 5·5 or 6·5.

When the PG (V) and PG (R) activities of filtrates were compared with pectin and polypectate as the substrates to be degraded, it was found that the *B. fabae* filtrates were about two to three times as active on polypectate as on pectin by both methods of assessment. But the PG (V) activity of *B. cinerea* filtrates on polypectate was many times greater than on pectin, although the PG (R) activity on the two substances was about the same or somewhat greater on pectin than on polypectate. Thus, after 28 hr. the filtrates had caused about 80% hydrolysis of pectin, but only some 60% hydrolysis of polypectate as assessed on reducing groups. The pectin contained a certain proportion of 'ballast' material so this figure of 80% probably represents complete hydrolysis of polyuronides present because paper chromatography of the mixtures after 28 hr. revealed galacturonic acid and no intermediate uronides, or other substances.

These results can be partly explained by the fact that the PG (V) activity is measured over the first few minutes of the reaction and during this period there are relatively many fewer glycosidic linkages liable to hydrolysis in the pectin as compared with the polypectate chains because of the esterification of the former. Later, the pectinesterase present in the culture filtrates causes more glycosidic linkages to become available so that with time the pectin becomes a more suitable substrate. However, this does not explain why ultimately the pectin is more extensively degraded than the polypectase.

(*c*) *Cellulase activity*. Incubation of filtrates from cultures of both fungi on CMC and wood cellulose, with wood cellulose at pH 5·5 for 5 days under toluene revealed no increase in reducing groups, so it was concluded that C 1 type cellulases were absent. *B. cinerea* cultures on wood cellulose and CMC had a relatively high Cx activity of 20–30 at pH 5·5 with the more highly polymerized CMC substrate. Activity was about a half of this when the less highly polymerized CMC was used.

In contrast, *B. fabae* cultures were almost inactive, although almost as much growth was made on CMC as on glucose. Presumably, Cx was secreted during growth but not in sufficient quantities to accumulate. This result has also been obtained with other fungi (Wood, 1961).

Another interesting fact which emerged from these experiments was that both fungi after growth on CMC, but not on glucose or wood cellulose, produced filtrates with relatively high PG (V) activity (about 20), at pH 5·5.

(*d*) *Effect of culture filtrates on bean tissue*. Sections 1 cm. long were cut from each of the three internodes of a number of bean plants at the four-leaf stage. They were immersed for 48 hr. at 25° C. in unheated and autoclaved filtrates, adjusted to pH 5·5

with citrate buffer (final 0·05 M), and obtained from cultures grown with glucose, pectic substances and celluloses as the main carbon sources; a few drops of toluene were added as a preservative. Sections in each of the autoclaved filtrates, in unheated glucose filtrates of both fungi, and unheated wood-cellulose filtrates from *B. fabae* cultures retained their original firmness. None of these filtrates had detectable PG or Cx activity. Each of the other filtrates had PG and Cx activity and affected the sections in the following ways (C—collapsed, S—generally soft, F—firm, B—brown, G—green).

Table 3. *Effect of culture filtrates on stems*

	Internode		
	1 (oldest)	2	3 (youngest)
	B. fabae		
Pectin	FG	SB	SB
Polypectate	CB	CB	CB
CMC	FG	SB	CB
	B. cinerea		
Pectin	FG	FG	SB
Polypectate	CB	CB	CB
CMC	FB	SB	CB
Wood cellulose	SB	CB	CB

The older internodes were more resistant to the action of the filtrates than the younger ones, and the polypectate filtrates were more active on the stems than were the other filtrates. Appropriate controls without toluene showed that this preservative had no effect on the macerating enzymes, but it did remove the pigment from the sections.

From these results it was not possible to assess the relative importance of PG and cellulose in macerating the stem tissues because each filtrate contained both types of enzymes, but it may be significant that the *B. cinerea*–wood cellulose filtrates in which the Cx/PG ratio was high had almost as much effect on the stems as the polypectate filtrates with a much lower Cx/PG ratio.

(*e*) *Effect of leaf extracts on enzyme secretion.* These experiments were confined to *B. fabae*, which was grown in the way described above except that leaf extracts were added to some of the media so that 50 ml. medium contained extract corresponding to 0·5 g. fresh-weight leaves. The leaf extract was added to the basal medium, which was allowed to stand for 5 hr. at 20° C., and then polypectate or pectin solutions were added to a final concentration of 1 %. Solutions containing leaf extracts turned brown very quickly on addition of the pectic substances; the mixture with polypectate became intensely dark brown, but the one with pectin did not darken as much. Controls without leaf extracts were pale yellow in colour. All media were adjusted to pH 5·0 before inoculation, and the growth and enzyme activity after growth for 7 days at 25° C. are shown in Table 4.

Growth of mycelium on pectin was increased by 60 %, and on polypectate by 100 % by adding leaf extract, but the PG (V) activity of the polypectate filtrates which was quite high in the absence of leaf extracts was very low when it was added. The PG (V)

activity on pectin was low in all filtrates but again the leaf extract reduced activity. More will be said of this later.

Segments, 1 cm. long, from the stems of bean plants at the four-leaf stage were immersed in each of the filtrates after these had been adjusted to pH 5·5. After incubation for 48 hr. under toluene at 20° C. each of the filtrates without leaf extract had completely disintegrated the segments and the residues were black in colour. The filtrates with leaf extract had only caused the segments to become slightly soft with the cut ends discoloured brown. The leaf extract had, therefore, either reduced secretion of, or inactivated, the enzymes responsible for degradation of pectic substances *in vitro*, and those which macerate bean stems.

Table 4. *Effect of leaf extracts on growth and enzyme secretion*

Main carbon source	Leaf extract	Dry wt. (mg.) mycelium	PG (V) activity	
			Polypectate	Pectin
Pectin	−	50	24	1·7
Pectin	+	82	1	0·5
Polypectate	−	71	14	1·7
Polypectate	+	142	1	0·8

II. *The browning reactions of leaves*

There can be little doubt that the typical colour of chocolate-spot lesions is a reaction of the host tissue to damage to the cells caused by *B. fabae* or *B. cinerea*, because lesions very similar in appearance can be produced quite readily and in a variety of ways by damaging leaf tissue mechanically or chemically. It was important, therefore, to know something about the processes leading to discoloration, and the following experiments were done with this in view.

(*a*) *Browning of leaf extracts.* Water extracts of leaves prepared as described above retain their green colour for some time but under certain conditions discolour rapidly if polypectate is added. Leaves of different ages give extracts which discolour at different rates, as was shown by an experiment in which one volume of a 1 % solution of polypectate was added to 10 volumes of extracts of leaves 1 (oldest) 2, 3 and 4 (youngest). After 15 min. extract of leaf 4 was green and almost unchanged in colour, extract of leaf 3 was green at the bottom of the tube but gradually became red-brown in colour at the top, whereas extracts of leaves 2 and 1 were uniformly an intense red-brown in colour. In the absence of polypectate none of the extracts changed colour.

This discoloration was prevented if extracts were boiled for 5 min. before the polypectate was added, and the rate of discoloration in unboiled extracts was affected by pH with optima in the range 4·5–5·0 depending on the type of buffer used. The rate of discoloration fell much more rapidly at pH values above the optimum than at lower values.

It was also found that pectin, polygalacturonic acid, and CMC acted in the same way as polypectate.

From these results and those of other workers it may be presumed that the discoloration of leaf extracts follows the action of phenolases on phenols present in the leaves, but that in water extracts very little of the phenolase is present in an active

state. The addition of polypectate or other polymers increases greatly the proportion active/inactive phenolase in the way described by Kenten (1957, 1958) for other agents. Because of the significance of discoloration, and of pectic enzymes, having substances such as polypectate as substrates, in the formation of lesions, the activation of the phenolases present in leaves was now studied in some detail. The general procedure was to prepare dialysed leaf extracts in water, to activate the phenolase in one way or another, and then to determine the phenolase activity of the final solution, generally with catechol as substrate. Some of the more important results obtained are given below.

(*b*) *Effect of* pH *on phenolase activity.* Leaf extracts which had been activated with polypectate at pH 4·5 were allowed to oxidize catechol at a number of pH values. The extinction changes over the period 20–200 sec. after adding the substrate were as follows: pH 4·0–5·8, 4·5–5·2, 5·0–6·1, 5·5–8·8, 6·0–10·6, 6·5–12·6 and 7·0–13·2. In subsequent experiments activity was measured at pH 7·0.

(*c*) *Relation between amount of phenolase and activity.* After activation in the way described above different volumes of leaf extract produced these extinction changes in the period 20–200 sec. from the beginning of the reaction: 0·3 ml.—15·3; 0·25 ml.—13·4; 0·2 ml.—11·7; 0·15 ml.—8·6; 0·1 ml.—5·4; 0·05 ml.—2·9. Over this range, therefore, there was a linear relation between volume of extract and activity. In later experiments 0·2 ml. leaf extract was used.

(*d*) *Activation after different periods of incubation.* Phenolase in leaf extracts was activated at pH 4·5 with polypectate for different periods between 0 and 18 min. Activity increased with time to a maximum after 10 min. Subsequently, activation mixtures were allowed to stand for 15 min. before estimating activity.

(*e*) *Effect of* pH *on activation.* Leaf extract was activated with NaDSS at a final concentration of 4 mM, or polypectate at a final concentration of 1·4 mg./ml. The phenolase activity of the extracts afterwards is shown in Table 5.

Table 5. *Effect of* pH *on activation*

		Phenolase activity						
Activator	pH 3·5	4·0	4·5	5·0	5·5	6·0	6·5	7·0
Buffer only	0·5	0·5	0·9	0·4	0	—	—	—
Buffer + polypectate	6·4	10·7	11·6	7·6	0·9	—	—	—
Buffer + NaDSS	—	0·7	5·2	12·2	17·2	12·7	8·8	4·5

There was a well-marked optimum with NaDSS at pH 5·5, but at this pH polypectate was almost ineffective although at pH 4·5, the optimum value, activity was high and about two-thirds of the highest obtained with NaDSS. Polypectate is, therefore, a somewhat less effective activator than NaDSS, which was the best of a number of substances used by Kenten (1957, 1958).

(*f*) *Effect of concentration of activators.* Solutions of polypectate, polygalacturonic acid and pectin were made by carefully and slowly adding 1 g. of each to cold water stirred gently with a magnet. The pH was adjusted to 4·5, the volume made up to 100 ml., and the solutions were stored under toluene overnight at 4° C. before use. The relative viscosities of 0·5 % solutions at pH 4·5 were—polypectate 7·4, poly-

galacturonic acid 1·5, and pectin 3·6. The activity of leaf extracts after activation by different quantities of these substances is given in Table 6.

Each of the substances had the same order of activity, but pectin was somewhat less effective than the other two. Each of the substances retained a good part of its activity after considerable dilution and in this respect differed from the anionic wetting agents used by Kenten (1958).

Table 6. *Activation of phenolase by different substances at* pH 4·5

	Phenolase activity (conc. activator mg./ml.)					
Activator	$1{\cdot}4$	$1{\cdot}4 \times 10^{-1}$	$1{\cdot}4 \times 10^{-2}$	$1{\cdot}4 \times 10^{-3}$	$1{\cdot}4 \times 10^{-4}$	Control*
Polypectate	13·3	11·4	9·6	7·1	4·2	17·5
Polygalacturonic acid	13·5	11·9	10·1	8·5	5·1	17·5
Pectin	10·1	8·3	8·0	4·7	3·6	18·1

* Activation given by 4 mM NaDSS at pH 5·5.

These substances were also tested after they had been acted upon by *B. fabae* culture filtrates for relatively short periods, up to 90 min., or for longer periods, 24 hr. or more, after which most of the molecules would have been degraded to low molecular weight compounds, mostly galacturonic acid. After the substances had been degraded, the solutions were held at 100° C. for 5 min. before adding to the leaf extracts; it was found later that this was unnecessary because *B. fabae* culture filtrates alone had little effect on activation.

The degraded substances were slightly less active than the parent compounds, but the differences did not exceed 10% of the original activity.

Mono-galacturonic acid was found to be about half as active as the various polymers, and it would seem probable that these polymers are activators because of their carboxyl groups which make them strongly negative colloids in solution. They may act, therefore, in ways similar to the anionic detergents used by Kenten (1958).

(*g*) *Browning reactions in leaves of different ages.* It has been mentioned above that extracts from older leaves discolour more rapidly than do those from younger leaves, and in an earlier paper, Deverall & Wood (1961) showed that lesions are produced more readily on older leaves. The low rate of browning in young leaves could be caused by one or more of a number of factors and the following experiments were designed to find out which were operative.

(1) To see whether or not the concentration of phenolase was a limiting factor in young leaves, extracts of leaves 1–4 were dialysed to remove naturally occurring phenols, activated with 4 mM NaDSS at pH 5·5 for 15 min., and then allowed to oxidize 3·5 mg. of catechol or DOPA at pH 7·0. The amount of discoloration of the solutions caused by each of the extracts was almost the same; the colour of DOPA solutions was about twice as intense as that of catechol solutions. The different leaves, therefore, had similar contents of phenolase, and the extracts of young leaves did not lack the substances which react with the quinones, produced by the oxidation of phenols, to give coloured products.

(2) The phenolase of a dialysed extract of leaf 4 (youngest) was activated with NaDSS and used to oxidize the phenols present in non-dialysed extracts of leaves 1–4.

The extinction changes over the period 20–200 sec. after the beginning of the reaction were leaf 4–0, 3–3·7, 2–21·9, 1–28·6. Discoloration in extracts of leaf 4 did not start until 12 min. after the beginning of the reaction, with leaf 3 there was a much shorter lag of 170 sec., but only with leaf 1 was there no lag period. This experiment was repeated with the difference that 3·5 mg. of DOPA was added to the extract of each leaf before the dialysed leaf extract was added. Now, there was no lag phase with extracts of leaves 1 and 2, with leaf 3 the lag was 80 sec., and with leaf 4, 140 sec.

It seemed, therefore, that in the younger leaves there were dialysable substances which prevented the accumulation of coloured end products, until the system containing these substances had 'run down'. Systems are known which prevent further reactions with quinones by reducing the quinones to phenols (James, 1953).

III. *Inactivation of PG by products of phenolase action*

From the results given above it was known that the fungi causing chocolate spot secreted chain splitting pectic enzymes in appropriate media, and that pectic substances were efficient activators of the latent phenolase present in bean leaves. It was also found that leaf extracts greatly reduced the secretion of pectic enzymes when added to media which were otherwise suitable, and it was known from work with a variety of other fungi (Wood, 1960), that pectic enzymes could be inactivated by substances formed following the oxidation of phenols. This sort of substance will be abundant in chocolate spot lesions, so the inactivation of the pectic enzymes of the pathogens by leaf extracts and other preparations was studied in the following ways.

(*a*) *Inactivation by leaf extracts.* Water extracts of leaves of mixed ages had no PG (V) activity, but increased the viscosity of polypectate solutions (finally 0·5 %) when added at the rate of 0·2 g. fresh-weight leaf in 10 ml. solution. This ability to increase the viscosity of polypectate solutions disappeared if the extract were frozen overnight at −20° C. before use, so this type of extract was used subsequently.

Cell-free culture filtrates were mixed with leaf extracts, or with water, 24 hr., or immediately before adding to polypectate solutions for determination of PG (V) activity. Mixtures finally contained extract corresponding to 0·2 g. fresh-weight leaf per 10 ml., and were at pH 5·5. The PG (V) activities are given in Table 7.

Table 7. *Effect of leaf extracts on PG (V) activity*

	PG (V) activity			
	B. fabae		*B. cinerea*	
Incubation time (hr.)	Water	Leaf extract	Water	Leaf extract
0	12	14	25	25
24	3	0	16	7

A large part of the activity of *B. fabae* filtrates was lost after 24 hr. incubation in water, but the filtrates were *completely* inactivated by the leaf extracts in the same period. *B. cinerea* filtrates also lost some activity on dilution with water and incubation, but there was a considerably greater loss when leaf extracts were used.

This experiment was repeated with an additional series of treatments in which NaCN was added to the culture filtrates at a final concentration of 0·002 N. Cyanide had no direct effect on the activity of *B. cinerea* PG but reduced the inactivation caused by leaf extract by about 50 %. It was not possible to assess the effect of NaCN on the inactivation of *B. fabae* filtrates by leaf extract because alone it caused complete inactivation after 24 hr. The darkening of the filtrates containing leaf extracts was almost completely prevented by cyanide.

Tests with NaCl and NaCN showed that it was the CN' ion which inactivated *B. fabae* PG. This was another difference between the enzymes of the two organisms.

When the effects of extracts of leaves 2 and 4 were compared, it was found that those from leaf 2 caused considerably more inactivation than those from leaf 4 (youngest). In fact, although leaf 4 extracts did cause some inactivation of *B. fabae* PG, they had no measurable effect on *B. cinerea* PG.

Further experiments in which the effect of incubating leaf extracts with untreated and autoclaved filtrates from cultures of both fungi showed conclusively that most of the dark brown discoloration was produced by the action of *fungal* phenolase on the phenols of the host tissue.

(*b*) *Inactivation by products of oxidation of catechol or DOPA.* Dialysed phenolase from leaf 1 was activated with $1{\cdot}4 \times 10^{-1}$ mg./ml. CMC at pH 4·0 for 15 min. and allowed to oxidize 3·5 mg. catechol or DOPA in 7·0 ml. at pH 7·0 for 70 min. DOPA solutions were then red in colour, catechol solutions were yellow, and the controls in which boiled phenolase had been used were colourless. Equal volumes of these solutions and *B. fabae* and *B. cinerea* culture filtrates were mixed, incubated for different periods under toluene at 20° C., and then PG (V) activity was measured. The results are shown in Table 8.

Table 8. *Inactivation of PG by product of oxidation of catechol and DOPA*

	PG (V) activity			
	Control		Oxidized	
Incubation period (hr.)	Catechol	DOPA	Catechol	DOPA
	B. fabae			
1	7	6	4	1
20	1	1	1	1
40	1	1	1	1
	B. cinerea			
1	25	25	25	25
20	7	16	6	11
40	1	10	2	11

After 1 hr., *B. fabae* PG had become almost inactivated in the presence of oxidized DOPA, and about half of the activity was lost in solutions of oxidized catechol. With longer periods of incubation each of the mixtures lost most of their activity, but from the design of this experiment it was not possible to say how much of this further inactivation had been caused by dilution and how much by the continued action of oxidation products.

B. cinerea PG was unaffected after incubation for 1 hr. with oxidation products, but after 20 hr. products of catechol oxidation caused about 75% inactivation and after 40 hr. had almost completely inactivated the filtrates. The products of DOPA oxidation had far less effect, particularly after prolonged incubation.

The absence of differences between the controls and the other treatments undoubtedly was caused by the oxidation of the phenols by fungal phenolase because the controls had become coloured 1 hr. after the components had been mixed.

This experiment revealed another difference between the PG of the two fungi; *B. fabae* PG was rapidly affected by products of DOPA oxidation, whereas *B. cinerea* PG was only slightly affected by these products, although it was inactivated slowly by products of catechol oxidation.

The rapid inactivation of *B. fabae* PG was studied in an experiment with the following treatments.

(1) Culture filtrate incubated with oxidized DOPA prepared as above.

(2) Culture filtrate incubated with buffer at pH 7·0.

(3) Culture filtrate mixed with oxidized DOPA immediately before measurement of activity.

(4) As 3 with buffer at pH 7·0 instead of oxidized DOPA.

The results are given in Table 9.

Table 9. *Inactivation of* Botrytis fabae *PG by oxidized DOPA*

	PG (V) activity			
	Incubated with		Mixed immediately with	
Incubation period	Buffer	DOPA	Buffer	DOPA
0	20	20	20	20
10 min.	20	11	20	20
30 min.	16	4	20	20
60 min.	14	1	20	20
120 min.	14	1	20	20
19 hr.	8	0·5	20	20

The rate of inactivation of *B. fabae* PG when incubated with buffer was substantial but less rapid than had been obtained in other experiments. There was no inactivation when the PG was incubated without dilution, and when PG was mixed with oxidized DOPA immediately before activity was measured. But in the presence of oxidized DOPA inactivation was rapid; there was almost 50% inactivation after 10 min., 80% inactivation after 30 min., and almost complete inactivation after 60 min.

DISCUSSION

B. cinerea and *B. fabae* produced in cultures on appropriate media enzymes which rapidly degraded various pectic substances, and which also degraded soluble cellulose derivatives. They also produced enzymes which readily macerated sections of bean stems and caused the tissue to become black in colour. An attempt will now be made to assess the importance of these enzymes in the formation of chocolate-spot lesions. In the first phase of the disease the lesions are discrete, limited in size and there is not

much evidence of extensive breakdown of tissue so it would seem that even if the pathogens did produce cell-wall degrading enzymes at this stage, they are not produced abundantly and have little effect on the tissue after they have been secreted. Some of the results which have been obtained may explain, in part, how this happens, because leaf extracts greatly reduced the secretion of pectic enzymes when added to culture media which otherwise were suitable, and products formed, following the oxidation of phenolic substances such as might be found in bean leaves, rapidly reduced the activity of filtrates containing pectic enzymes. Also, pectic substances similar to those present in plant tissues, or to those which would be produced by the action of pectic enzymes on the cell wall, activate the phenolase in the bean leaves which otherwise would remain inactive. The sequence of events in the formation of the restricted type of chocolate spot lesion might, therefore, be somewhat as follows. The fungi infect the leaf tissue and begin to secrete cell-wall degrading enzymes, which act upon the cell wall to produce soluble derivatives. These stimulate the secretion of more enzyme, and also provide carbon sources for the further growth of the pathogen. The cells invaded by the pathogen, and adjacent to it, are killed, possibly by the direct action of the cell-wall degrading enzymes, and the latent phenolase of the cells becomes associated with soluble pectic substances which activate the phenolase, and this in turn acts upon phenols released by the death of the cells. The product of these reactions inactivate the pectic enzymes already secreted by the pathogen, and these interactions rapidly have a cumulative effect so that the growth of the lesions is prevented because cell-wall degrading enzymes are not produced in sufficient quantities to leave a balance which will continue to act upon unaffected leaf tissue.

Although this hypothesis is supported by some of the results of the experiments described above, other results are not readily accommodated by it. For example, it has been shown that lesions develop more readily on old than on young leaves, and that the latter contain a system which delays the formation of coloured products following the oxidation of phenols. This might explain why fewer and smaller lesions develop, but, on the other hand, the fact that less of the coloured products are present would imply less inactivation of pectic enzymes and greater degradation of tissue. Also, solutions of pectic substances did not cause lesions on leaves rubbed with a mild abrasive although under the same conditions solutions of NaDSS did so. But this is possibly explained by the fact that the expressed sap of leaves has a pH of 6·0–7·0, and in this range pectic substances do not activate the latent phenolase of the leaves; also, when the fungus initiates a lesion it is probable that the pH of the tissue is reduced below that of healthy leaves by the action of pectinesterase on the pectinic acids of the cell wall.

At present, little is known about the mechanisms by which pectic and cellulosic substances activate the latent phenolase, but it may be similar to that suggested by Kenten (1958) for substances such as NaDSS, and which corresponds generally with explanations of the activation of enzymes by heat proposed by Swartz, Kaplan & Frech (1956). In the inactive state phenolase is supposed to be bound by salt linkages to an inhibitory protein, and activation involves the removal of the protein from the complex by reactions between the anionic activators and cationic groups of the protein. Differences in the behaviour of the anionic detergents used by Kenten (1958)

and substances such as pectinic acids probably depend on factors such as the effect of pH on dissociation of the activating substances, and differences in molecular size and shape, e.g. figures of the order of 10^2 for the wetting agents and 10^5 for linear pectinic acids and pectates. There are few reports on naturally occurring activators of enzyme systems, or on inactive enzymes in plant cells. But it is likely that other enzymes less readily detectable than phenolase are present in a similar state, and the activation of such enzymes by substances produced by the action of a pathogen might be a factor in pathogenesis which up to now has not been considered.

It has been stated earlier that, under appropriate conditions, *B. fabae* does not remain confined to discrete, small lesions, but spreads 'aggressively' to occupy large parts of the leaf; *B. cinerea* does this much less readily. No direct evidence to explain these differences in behaviour has been obtained, but it can be suggested that in the particularly favourable conditions of the aggressive phase pectic and other cell-wall-degrading enzymes are produced at a rate in excess of their inactivation by oxidation products so that there remains a surplus at the edge of the lesion which, therefore, continues to advance. If this were so then it should be possible to obtain cell-wall degrading enzymes from the edges of aggressive lesions but not from the smaller type of lesion. This remains to be done.

Finally, the different behaviour of *B. fabae* and *B. cinerea* will be considered. In the first place, there can be no doubt that *B. fabae* is a much more effective pathogen of beans than is *B. cinerea*. Secondly, the two fungi behave differently *in vitro* in the following ways: (*a*) *B. cinerea* grew far more rapidly than *B. fabae* in culture media on a variety of carbon sources; (*b*) *B. cinerea* produced PG, pectinesterase, and cellulase on appropriate media, whereas *B. fabae* culture filtrates had very little cellulase activity and less PG and pectinesterase activity under the same conditions; (*c*) *B. fabae* culture filtrates rapidly lost PG activity on dilution, whereas *B. cinerea* retained most of their PG activity under the same conditions; (*d*) *B. fabae* PG, but not *B. cinerea* PG was rapidly inactivated by the CN′ ion; (*e*) *B. fabae* PG was readily inactivated by products formed following oxidation of DOPA, but was less affected by products of catechol oxidation, whereas *B. cinerea* PG was affected in the opposite way. None of these differences would explain why *B. fabae* is a better pathogen than *B. cinerea*. On the contrary, some of them would be good explanations if the opposite were true. This aspect of the chocolate spot problem therefore remains quite unexplained.

REFERENCES

Deverall, B. J. & Wood, R. K. S. (1961). Infection of bean plants (*Vicia faba* L.) with *Botrytis cinerea* and *Botrytis fabae*. *Ann. appl. Biol.* **49**, 461.

James, W. O. (1953). Oxidation mechanisms. In *Plant respiration*, pp. 198–9. Clarendon Press, Oxford.

Jansen, E. F. & MacDonnell, L. R. (1945). Influence of methoxyl content of pectic substances on the action of polygalacturonase. *Arch. Biochem.* **8**, 97.

Kenten, R. H. (1957). Latent phenolase in extracts of broad bean (*Vicia faba* L.) leaves. I. Activation by acid and alkali. *Biochem. J.* **67**, 300.

Kenten, R. H. (1958). Latent phenolase in extracts of broad bean (*Vicia faba* L.) leaves. 2. Activation by anionic wetting agents. *Biochem. J.* **68**, 244.

Leach, R. (1955). Recent observations on the *Botrytis* infection of beans. *Trans. Brit. mycol. Soc.* (Abstr.), **38**, 171.

Ogilvie, L. & Munro, Moira D. (1947). Occurrence of *Botrytis fabae* Sardiña in England. *Nature, Lond.* **160**, 96.

Swartz, M. N., Kaplan, N. O. & Frech, Mary E. (1956). Significance of 'heat-activated' enzymes. *Science*, **123**, 50.

Wood, R. K. S. (1960). Pectic and cellulolytic enzymes in plant disease. *Ann. Rev. Plant. Physiol.* **11**, 299.

Wood, R. K. S. (1961). *Verticillium* wilt of tomatoes: the role of pectic and cellulolytic enzymes. *Ann. appl. Biol.* **49**, 120.

Ann. appl. Biol. (1961), **49**, 488–496
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Fruit-spot ('speckle') of Jamaican bananas caused by *Deightoniella torulosa* (Syd.) Ellis

IV. Further observations on spore dispersal

By D. S. MEREDITH
Banana Board Research Department, Kingston, Jamaica, B.W.I.

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SUMMARY

Using an automatic volumetric spore trap, variation in the atmospheric spore content of *Deightoniella torulosa* (Syd.) Ellis, the cause of banana fruit 'speckle', has been studied in a banana plantation during the period September 1960–February 1961. Dry weather was characterized by relatively low daily mean spore concentrations of 50–200 spores/m.^{3} of air. Much higher concentrations (2000–7000 spores/m.^{3}) were recorded shortly after rainfall or periods of under-tree irrigation.

Considerable reduction in concentration occurred after the removal from the plantation of decaying leaves and other banana 'trash', such tissues being the major source of inoculum of *D. torulosa*. Similarly, spore counts in trash-free plantations were consistently lower than those in 'dirty' ones. Although the evidence is not conclusive, since it was not possible to provide controls, it seems likely that good plantation hygiene is a major factor in reducing the air spore content of the fungus.

In the plantations, the large conidia of *D. torulosa* have a short air-borne phase, probably because sedimentation and/or deposition rates are high. This results in relatively short horizontal dispersal distances.

INTRODUCTION

The banana industry of Jamaica is subject to a certain amount of wastage of fruit due to a fruit-spot disease ('speckle') caused by the fungus *Deightoniella torulosa* (Syd.) Ellis (Meredith, 1960*a*; 1961*a*, *b*, *c*). Speckle does not adversely affect the ripening behaviour or eating qualities of bananas but, nevertheless, the unattractive appearance of diseased fruit considerably reduces its market value and it is usually rejected in the tropics. Wastage caused by speckle has been reported in other Caribbean islands, notably Haiti (Willis, 1960), and certain of the Windward Islands (Loos, 1960; Spence, 1960). In 1953 and 1954, speckle resulted in many thousands of bunches being wasted in Honduras and Guatemala (Anon., 1956; Meredith, 1961*e*). In Central America attempts to control speckle have been made by covering young bunches of fruit with plastic bags (Anon., 1958). In one 6-month period, during which the heaviest annual rainfall occurred, this form of bunch protection effected moderate to good control. Removal of the bag before harvest commonly resulted in increased disease severity, especially if there was heavy rainfall. Similar bagging experiments

carried out in Jamaica indicated some control of speckle (Leach, 1959). However, because of excessive material and labour costs, it is unlikely that bagging will be adopted widely in Jamaica.

The possibility of controlling speckle by phytosanitary methods has been suggested by Meredith (1960*b*, 1961*c*). *D. torulosa* is a common saprophyte on decaying pseudostems, leaves, bracts, fruit and other banana 'trash' inside the plantation. Under wet conditions sporulation is abundant and, under conditions of rapidly decreasing vapour pressure, conidia are violently discharged (Meredith, 1961*d*), thus becoming air-borne. It was thought that periodic removal of trash might decrease inoculum sources to such an extent that speckle ceases to be of commercial importance. In the present paper an attempt has been made to relate the atmospheric spore content of *D. torulosa* to plantation hygiene and to other factors, namely rainfall, irrigation and wind damage.

METHODS

Trapping and scanning procedure

An automatic volumetric spore trap (Hirst, 1952) was set up inside a banana plantation with its orifice situated 3 m. above ground level; air was sampled at the rate of 10 l./min. Slides were prepared and mounted as described by Hirst (1953) and changed at 09·00 hr. E.S.T. each day. Spores were counted on cross traverses 40 μ wide and 4 mm. apart, representing 2-hourly intervals; estimates of numbers of spores/m.^{3} of air were then calculated. Daily mean concentrations were calculated by averaging the 2-hourly estimates for the period 24·00–24·00 hr. each day. No corrections for variation in efficiency of the trap were made, so that all spore concentrations quoted are underestimates (Hirst, 1953).

Except for occasional failures, the trap was operated continuously during the period 1 September 1960–18 February 1961.

Plantation conditions

The plantation selected for special study was a 'plant' crop and was situated on the lowland plains in St Catherine; a brief description has been given elsewhere (Meredith, 1961*c*) but it is desirable to recall certain features. Plants were predominantly of the Lacatan variety and spaced at the rate of approximately 900/acre. The method of irrigation was to pump water along portable metal pipes from which it was sprayed through elevated rotating nozzles (under-tree irrigation). Each irrigation period was approximately 48 hr. in duration and, since water is sprayed several feet into the air, collapsed leaves hanging from pseudostems (and occasionally hanging bunches of fruit) were thoroughly wetted.

In considering the air-sampling data, it is convenient to distinguish three successive periods according to plantation and weather conditions.

(1) 1 September–11 November. Initially most plants were bearing several collapsed, decaying leaves; only a few plants had flowered. Later, as fruit approached maturity, harvesting was carried out at weekly or fortnightly intervals and a few dead leaves were removed each time for use in packing. During the harvesting operation, the

pseudostem is severed about 4 ft. above ground and the felled portion is chopped into small pieces which are left in the plantation.

Rainfall was recorded on 28 days and amounted to a total of 24·63 in. The mean daily maximum temperature, which generally occurs around noon to 14.00 hr., was 86·9° F. and the mean minimum temperature was 70·5° F.

(2) 12 November–11 December. De-trashing was commenced on 12 November in an attempt to improve plantation hygiene. All dead and dying leaves were severed close to the pseudostem and old pseudostem stumps were cut down to ground level. The appearance of plants before and after detrashing is shown in Plate figs. 1, 2. All trash in an area approximately 100 m. in diameter, at the centre of which the trap was situated, was removed from the plantation and burnt. The cleared zone was then lightly harrowed in order to bury small pieces of trash. In regions beyond the cleared zone, cut down trash was left on the ground.

On 3 December a moderate to strong north-westerly wind (the 'dry norther') developed and persisted for nearly 5 days. More than 40 % of the crop in the experimental plantation was severely damaged, injury commonly being in the form of doubling-over of the pseudostem just below the crown (Plate fig. 3). On 10 December wind-blown plants were cut down, chopped into small pieces and left on the ground to decay.

Rainfall occurred on 12 days, the total being 3·22 in. The mean daily maximum and minimum temperatures were 90·4 and 70·6° F., respectively.

(3) 12 December–26 January. This was a predominantly dry period (3·94 in. rain) and plant growth was relatively slow. Mean daily maximum and minimum temperatures were 91·7 and 67·3° F. Although the plantation floor was littered with trash, the rate of decay was extremely slow. Because of earlier wind damage and successive harvestings the majority of plants at the conclusion of sampling were young followers, not yet having flowered.

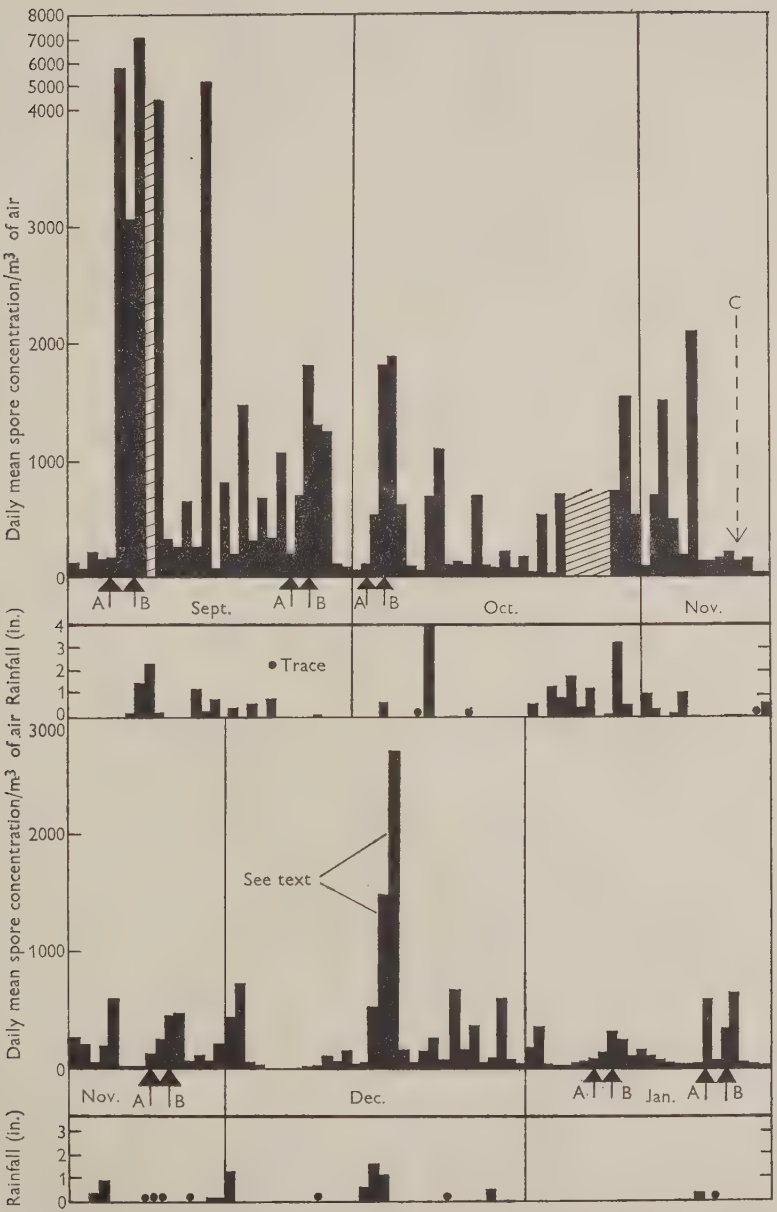
Meteorological records

Daily records of rainfall, temperature and humidity were obtained from instruments situated in a small clearing near the trap.

RESULTS

In Text-fig. 1 daily mean concentrations of *D. torulosa* are plotted for the period 1 September 1960–26 January 1961, and are related to total daily rainfall and to irrigation. Results for the period 1 September–5 October have been discussed in detail elsewhere (Meredith, 1961*c*), where it was noted that *D. torulosa* exhibits a regular diurnal periodicity. Rapid liberation of conidia starts at about 07.00 hr. and reaches a peak near 08.00 hr. After this there is a rapid decrease to a low concentration which is maintained throughout the afternoon and night. Similar periodicity was evident during the period 5 October–26 January, the only exceptions being 5–8 December when no spores were trapped, and 22 and 29 October, 2 and 9 November when the peak concentration occurred between 10.00 and 12.00 hr.

In general, days having rain, or on which irrigation was carried out, were followed



Text-fig. 1. Daily mean concentration of *D. torulosa* conidia related to rainfall, under-tree irrigation and plantation hygiene during period 1 September 1960–26 January 1961: arrows A indicate commencement of irrigation, B cessation; de-trashing carried out on 12 November (arrow C); hatching indicates that trap was not working.

on successive days, by varying increases in daily mean concentration. This is due to increased sporulation of *D. torulosa* on trash after wetting (Meredith, 1961*c*). Before de-trashing was carried out on 12 November, daily mean concentrations exceeding 1000, 2000, 3000 and 5000 spores/m.^{3} were recorded on 16, 6, 5 and 3 occasions, respectively. In contrast, the highest value recorded in the same plantation after de-trashing was only 750 spores/m.^{3} In Table 1 the seventeen highest early morning peak concentrations recorded both before and after de-trashing are compared. Before de-trashing, 0·32, 1·0 and 1·22 in. rain resulted in peak concentrations of 14,190,

Table 1. *Relation between maximum 2-hourly concentration of* Deightoniella torulosa *conidia and rainfall or irrigation: seventeen highest counts recorded before and after de-trashing plantation*

Before de-trashing				After de-trashing			
Date	Spore concentration (no./m.^{3})	Rainfall on previous day (in.)	Irrigation on previous day	Date	Spore concentration (no./m.^{3})	Rainfall on previous day (in.)	Irrigation on previous day
8. ix. 60	72,335	0·11	+	2. xii. 60	7689	1·28	−
15. ix. 60	57,270	1·22	−	23. xii. 60	7000	Trace	−
10. ix. 60	49.130	2·32	−	20. i. 61	6510	0·14	−
6. ix. 60	47,124	Nil	+	16. xii. 60	6000	0·57	−
7. ix. 60	20,655	Nil	+	29. xii. 60	5852	0·50	−
6. xi. 50	19,090	1·00	−	23. i. 61	5808	Nil	+
26. ix. 60	18,117	Nil	+	19. xi. 60	5775	0·90	−
30. x. 60	17,061	3·15	−	26. xi. 60	5346	Trace	−
3. xi. 60	14,190	0·32	−	1. xii. 60	5280	0·13	−
19. ix. 60	14.190	0·48	−	25. xi. 60	4884	Trace	−
28. ix. 60	13.200	Nil	+	2. i. 61	3993	Nil	−
27. ix. 60	12,144	Nil	+	15. xi. 60	2838	0·50	−
23. ix. 60	11,517	0·80	−	24. xi. 60	2805	Trace	−
10. x. 60	8,745	4·12	−	22. i. 61	2505	0·05	+
23. x. 60	7,458	1·35	−	30. xi. 60	2442	0·10	−
17. ix. 60	7,381	0·28	−	10. i. 61	2409	Nil	+
9. x. 60	7,095	Trace	−	18. xi. 60	2145	0·31	−

19,060 and 57,270 spores/m.^{3} on 3 and 6 November and 15 September, respectively. After de-trashing, comparable quantities of rain—0·31, 0·90 and 1·28 in.—were followed by the relatively low concentrations of 2145, 5775 and 7689 spores/m.^{3} on 18 and 19 November and 2 December, respectively. It is possible that the low counts obtained after de-trashing were a direct result of improved plantation hygiene around the trap. However, this suggestion may be criticized on the basis that there appears to be no consistent relationship between the quantity of rain and the resultant spore concentration. For instance, before clearing away trash, 1·22 in. rain between 14.00 and 16.00 hr. on 14 September resulted in a count of 57,270 spores/m.^{3} at 08.00 hr. on the following day, whereas 1·35 in. at a corresponding time on 22 October gave only 7458 spores/m.^{3} at 08.00 hr., 23 October. Other similar examples may be drawn from Table 1. It may be argued, therefore, that the relatively low counts obtained after de-trashing were coincidental and not necessarily related to plantation hygiene.

Successive periods of under-tree irrigation resulted in varying increases in spore concentration. The relatively large increases accompanying irrigation in September

and October were probably a result of wetting decayed leaves, thus favouring sporulation of *D. torulosa* which was, at that time, one of the most common fungi observed on such trash. The slight increase in spore concentration during the November irrigation was not unexpected since there was very little trash near the trap. Larger increases were anticipated in the cases of the two January irrigations for, as has been noted above, the plantation floor was littered with the remains of wind-damaged plants. However, repeated surveys revealed that *D. torulosa* was uncommon on this trash. Possibly the hot, dry days and relatively cool nights during the latter half of December and throughout January were factors limiting colonization by *D. torulosa*. Alternatively, trash lying on the ground might have been colonized by fungi other than those which normally invade collapsed leaves hanging from the pseudostem, and which competed successfully to the exclusion of *D. torulosa*.

The period 5–8 December was exceptional in that no conidia of *D. torulosa* were trapped. Extensive drying out of the plantation by the 'norther' undoubtedly resulted in conditions that were unfavourable to sporulation. (For four nights there was no dew formation and humidity never exceeded 78 %.) For the remainder of the sampling period the concentration of *D. torulosa* was relatively low, increasing slightly when there was rain or irrigation.

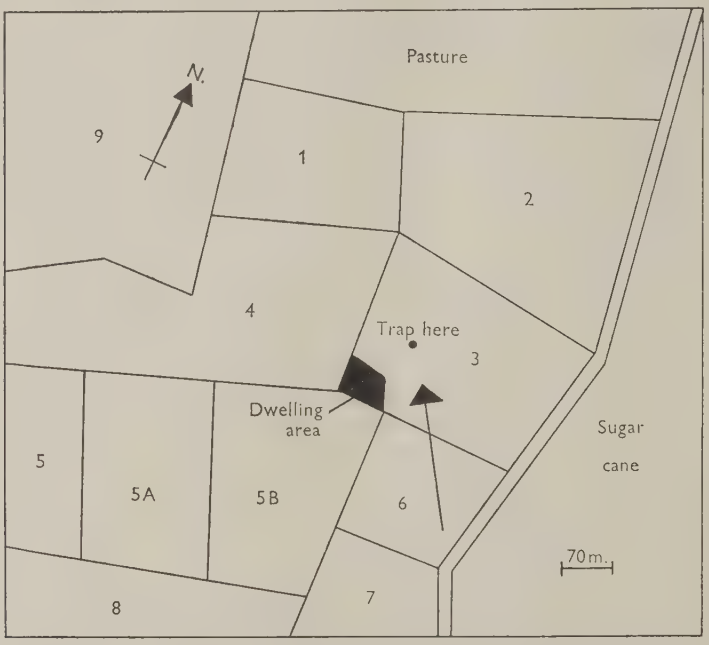
It is evident from Text-fig. 1 that the daily mean concentration decreased progressively during the sampling period. On 17 and 18 December, on which 1·53, and 1·20 in. rain, respectively, were recorded, the trap was operated in a nearby plantation in which there had been only slight wind-damage and from which no trash had been removed previously; the majority of plants were bearing several collapsed, decaying leaves. At 08·00 hr. on 17 and 18 December, concentrations of 10,593 and 24,360 spores/m.^{3}, respectively, were recorded (see Text-fig. 1). On 13 and 14 February, the trap was operated in yet another uncleaned plantation in which under-tree irrigation was in progress; early morning peak concentrations of 32,600 and 46,200 spores/m.^{3}, respectively, were recorded. Finally, on 16 February, the trap was replaced in the experimental plantation during an irrigation period. The highest 2-hourly concentration recorded was 7320 spores/m.^{3} These observations suggest that the progressive decrease in spore concentration observed in the experimental plantation was not due to any seasonal decrease in the population of *D. torulosa*, but that it was caused mainly by local plantation conditions.

Other exposures were made in some young plantations where plants were only 6–8 ft. high. Very little banana leaf trash was present and, as was anticipated, spore concentrations were low even after rainfall or irrigation.

DISTANCE OF DISPERSAL

The data in Text-fig. 1 provide some interesting information about dispersal distances under natural conditions. The map (Text-fig. 2) shows the position of the trap relative to surrounding banana plantations and other vegetation. During the current sampling period under-tree irrigation was carried out in plantations 7, 6, 5, 3, 4, 2 and 1, in that order; other plantations were surface-irrigated. Plantation 6 to the windward side of the experimental plantation was usually irrigated 2 days before the latter.

It might be expected that downward drift of conidia originating in plantation 6 would be indicated by an increased number of spores trapped some 100 m. to leeward. During each of the six irrigation periods in plantation 6, samples of trash were collected and Vaselined slides exposed near to collapsed, decaying leaves. There was evidence that large numbers of conidia of *D. torulosa* were liberated on the mornings in question but on no occasion was there any significant increase in spore concentration near the trap. Two possible explanations for this apparent short-range dispersal may be considered. First, since there is very little wind at the time of most rapid



Text-fig. 2. Map showing position of trap and vegetation during period 1 September–26 January. Prevailing wind direction indicated by lowermost arrow. Numbered areas were planted with bananas only.

spore liberation (between 07.00 and 09.00 hr.), it may be that, after violent discharge into the atmosphere, the large conidia of *D. torulosa* soon fall out under the influence of gravity. Correspondingly, dispersal distances would be relatively small. Evidence suggesting a short air-borne phase was obtained in earlier studies (Meredith, 1961*c*) where it was found that the concentration decreased from 60,000 to 3000 spores/m.^{3} between 08.00 and 10.00 hr. Alternatively it may be that horizontal drift in open sites is quite extensive even in relatively still air, but that the thick screen of vegetation formed by a banana plantation restricts spore drift owing to a high rate of deposition on leaves and other surfaces. Wind-tunnel tests have been planned in an attempt to gain some idea of sedimentation rates at different wind speeds.

DISCUSSION

This investigation has confirmed earlier studies (Meredith, 1961*c*) in showing that *D. torulosa* is a very common component of the air spora in several Jamaican banana plantations. Also, it is now clear that sporulation occurs throughout the year, being most abundant after wetting of the plantation by rain or under-tree irrigation.

The evidence suggesting that good plantation hygiene reduces the atmospheric spore content of *D. torulosa* is (1) that peak spore counts recorded before de-trashing greatly exceeded those recorded in the same plantation after de-trashing and, (2) that counts in non-de-trashed plantations were higher than those in old de-trashed or in young plantations, where only a few dead leaves were present. Unfortunately, only one spore trap was available and it was not possible to provide suitable controls for the various sampling experiments. Therefore this evidence is necessarily circumstantial, and it would be unwise to disregard the possibility that factors other than plantation sanitation had some effect in reducing the air spore content. Nevertheless, results to be published elsewhere indicate that good plantation hygiene results in a significant decrease in the severity of speckle, thus serving to strengthen the evidence presented here.

The author wishes to thank the United Fruit Jamaica Co. for providing the plantation facilities which made this investigation possible. He is indebted to Mr R. R. Chen for valuable technical assistance. This paper is published by permission of the Banana Board, Kingston, Jamaica, W.I.

REFERENCES

Anon. (1956). *Annu. Rep. Res. Dep. United Fruit Co., Boston, Mass.*, for 1955.

Anon. (1958). *Problems and progress in banana disease research.* Res. Dep. United Fruit Co., Boston, Mass.

Hirst, J. M. (1952). An automatic volumetric spore trap. *Ann. appl. Biol.* **39**, 257.

Hirst, J. M. (1953). Changes in atmospheric spore content: diurnal periodicity and the effects of weather. *Trans. Brit. mycol. Soc.* **36**, 375.

Leach, R. (1959). Report of plant pathologist. *Annu. Rep. Banana Board Res. Dep., Jamaica*, for 1958, 16.

Loos, C. A. (1960). Personal communication.

Meredith, D. S. (1960*a*). Banana fruit-spot ('speckle') caused by *Deightoniella torulosa* (Syd.) Ellis. *Nature, Lond.* **187**, 961.

Meredith, D. S. (1960*b*). Control of banana fruit-spot (*Deightoniella torulosa* (Syd.) Ellis). *Plant Dis. Reptr*, **44**, 808.

Meredith, D. S. (1961*a*). Fruit-spot ('speckle') of Jamaican bananas caused by *Deightoniella torulosa* (Syd.) Ellis. I. Symptoms of disease and studies on pathogenicity. *Trans. Brit. mycol. Soc.* **44**, 95.

Meredith, D. S. (1961*b*). Fruit-spot ('speckle') of Jamaican bananas caused by *Deightoniella torulosa* (Syd.) Ellis. II. Factors affecting spore germination and infection. *Trans. Brit. mycol. Soc.* **44**, 265.

Meredith, D. S. (1961*c*). Fruit-spot ('speckle') of Jamaican bananas caused by *Deightoniella torulosa* (Syd.) Ellis. III. Spore formation, liberation and dispersal. *Trans. Brit. mycol. Soc.* In press.

Meredith, D. S. (1961*d*). Spore discharge in *Deightoniella torulosa* (Syd.) Ellis. *Ann. Bot. Lond.*, N.S. In press.

MEREDITH, D. S. (1961*e*). Speckling of bananas in Central America caused by *Deightoniella torulosa* (Syd.) Ellis. *Trop. Agriculture, Trin.* In press.
SPENCE, J. A. (1960). Personal communication.
WILLIS, R. (1960). Personal communication.

EXPLANATION OF PLATE

Fig. 1. Lacatan banana plants bearing several collapsed, dead or dying leaves.

Fig. 2. Lacatan banana plants after pruning collapsed leaves.

Fig. 3. Bending of pseudostems caused by wind.

D. S. MEREDITH

(*Facing p.* 496)

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Germination and penetration studies on coffee rust (*Hemileia vastatrix* B. & Br.)

By R. W. RAYNER
*Coffee Research Station, Ruiru, Kenya**

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SUMMARY

Spore dispersal, germination, penetration and incubation-period studies on coffee rust (*Hemileia vastatrix* B. & Br.) in Kenya, East Africa, are described.

Evidence is produced that air-borne spores may be trapped effectively on the upper surfaces of leaves, thence to be liberated and transported to the undersurfaces of other leaves by rain splash.

Germination requires liquid water and was observed to occur in 2·6–4·7 hr. (medians) at 23° C., the minimum being 1 hr. Appressoria were formed in 6·5–8·5 hr. (medians) with a 5·3 hr. minimum. Germination is inhibited by light and in the field by the rapid evaporation of water droplets on the lower leaf surface which occurs during daylight. Light inhibits the growth of germ tubes that are less than 30 μ long and reduces rate of growth if they are longer; appressoria may continue to form.

In the field, appressorium formation and infection can occur between 10 p.m. and 8 a.m. If coffee trees are wet at dusk or rain falls before midnight, infection is probable and it is proposed that the number of occasions this occurs be used to forecast the severity of the annual rust maximum.

The median incubation period throughout the year varied from 4 to 7 weeks, increasing with low temperatures and dry conditions. A multiple regression of mean maximum and minimum temperatures on incubation period gave fair agreement between observed and computed values for Ceylon and Mysore. Susceptibility and incubation period were strongly affected by coffee variety and rust biotype, but not by age of leaf or crop size.

INTRODUCTION

In the Kenya Highlands annual attacks by *Hemileia vastatrix* on *arabica* coffee vary considerably in intensity. However, to control the disease by fungicides it is essential to apply a protectant spray before it is known whether a serious attack will develop. The writer has observed that unseasonable rains in January to February promote rust outbreaks later in the year. This relationship has recently been used to forecast outbreaks and issue warnings when sprays should be applied.

To improve the technique of forecasting the studies on germination and penetration described below have been carried out at the Coffee Research Station, Ruiru (altitude 5400 ft.).

Marshall Ward (1882) reported that at 24° C. *H. vastatrix* spores germinated in

* Present address: Commonwealth Mycological Institute, Kew, Surrey.

12–24 hr. and appressoria were formed in about 48 hr. At laboratory temperatures at Peradeniya, Ceylon a yellow area appeared on the infected leaf after about 14 days and sporing commenced 2–4 days later. Germination occurred only when spores were in contact with water, and penetration was via stomata, which are present only on the lower surfaces of the leaves. Using 'Vaselined' slides he demonstrated the spores to be airborne and considered that they alighted mainly on the upper surfaces of the leaves, thence to be washed round to the lower surface by rain.

Mayne (1933) in Mysore, southern India, confirmed many of Ward's findings. He found that light strongly inhibited germination, an observation also made by Bürk (1867).

EXPERIMENTAL

Spore dispersal

Ward (1882) showed that spores were liberated into the air and blown for considerable distances. To produce infection they must reach the stomata-bearing lower surface of the leaves. Using the spore-cloud method for depositing dry spores under a bell-jar (Morgan, 1960), the writer found none were deposited on the lower leaf surface. Spores deposited on the upper surface were dislodged by blowing only when a strong current of air was directed through a narrow jet placed about 0·3 mm. from a spore. If droplets of water were placed on the leaf the majority of spores floated to the surface. Thus, air-borne spores falling on leaves would be temporarily held on the upper surfaces from which they could be removed by rain.

The wetting of the undersurface of coffee leaves during rain storms was studied in the field. Some wetting resulted from raindrops landing directly on the undersurfaces when the leaves were disturbed by turbulent winds. Rain also flowed round the margins of the leaves on to the undersurface. However, most of the wetting was by raindrops rebounding from the top surfaces of lower leaves. Such splashes would carry with them spores deposited on these surfaces. That a drop of rainwater sometimes passes through a lesion, picks up spores and flows on across the leaf surface is suggested by the occasional occurrence of lines of small, young lesions running out from an old one.

Germination

Time required

With *in vitro* tests using hanging drops in Van Tieghem cells nil or very little germination was observed in diffused daylight, but it was abundant in the dark. In the light, when it did occur, the process took longer and the germ tubes were very stunted. In the dark at 19–20° C. it started after 2–3 hr., at 25° C. it took 2·3 to 3·5 hr., and at 30° C. it was slower and much reduced or nil.

Germination studies by earlier workers have been confined to glass substrata. To observe germination and appressorium formation on leaves and at various time intervals circular areas were marked on them and inoculated by stroking with a damp spore-bearing paint brush. After light spraying with water the inoculated leaves were placed in Petri dishes lined with damp filter-paper and were supported just clear of the paper by pinning their bases and tips to pillars of Plasticine. The inoculated areas were removed at intervals, one from each of the three leaves used, and examined for germination by drying them in a current of air and making a cellulose acetate cast as

described by Bennett & Furmidge (1956), but staining with Congo Red, which was found more satisfactory than Safranin.

In a preliminary test 98·8 % germination was found after 3 hr. The results of three further tests are shown in Fig. 1. Plotting probits suggested a somewhat skew distribution. Hence the results were summarized as median germination times which were 2·6, 3·7 and 4·7 hr. for the three tests. 5 % germination was estimated as occurring approximately at 1·0, 1·8 and 1·0 hr. and 95 % at 5·2, 6·2 and 9·8 hr. In one test, some germination was observable after only 1 hr. Hence, the process must start almost immediately the spores are wet and at 22° C. may be considered to be complete for practical purposes in about 7–10 hr.

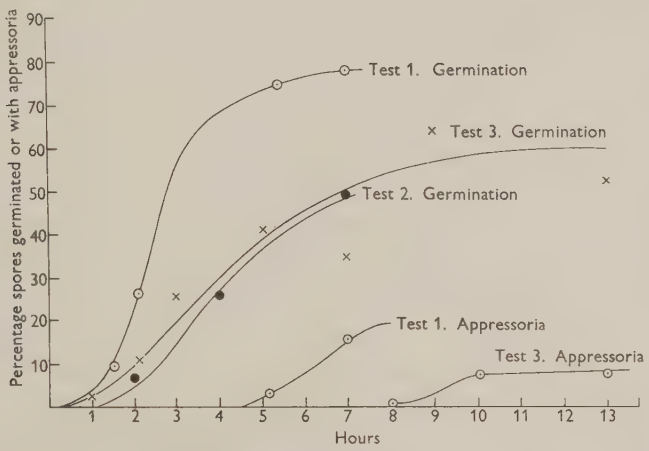

Fig. 1. Progress of germination and appressorium formation with time for *Hemileia vastatrix* spores on the undersurfaces of wet coffee leaves in the dark at 23° C.

Appressorium formation occurred in all tests, but was infrequent in the second. The earliest observed was at 5·3 hr. and probably did not occur in less than $4\frac{1}{2}$ hr. A median could only be estimated in one test and was about $8\frac{1}{2}$ hr. with 5 and 95 % points at roughly $7\frac{1}{2}$ and 10 hr., respectively. The maximum percentage of germinating spores producing appressoria was only 19·6 %.

The necessity for free water

The effect of high humidities on spore germination at constant temperature (25° C.) was investigated. Cover glasses for Van Tieghem cells, glass slides and leaves were sown with spores using Morgan's (1960) method. Slides and leaves were supported above moist filter-paper as before. In replicates the filter-paper was moistened with concentrated solutions of lead nitrate and sodium sulphite in contact with the solid phase in order to produce 98 and 95 % R.H., respectively. Wherever salt-free water was used, it condensed on the spore-sown surfaces and germination resulted. On the leaves appressoria were produced by up to 87·5 % of the germinated spores.

Germination was found on one of the leaves only at 95% R.H. but it was only incipient and abnormal. None occurred at 98% with either leaf or glass surfaces.

During other investigations on living leaves when liquid water disappeared from the surfaces inhibition of germination always resulted. It is concluded that high humidity alone is not sufficient to stimulate germination.

The effects of light

Studies by earlier workers on the effects of light have been confined to glass substrates. To determine whether germination could occur on inoculated leaves with full out-door illumination a few were sprayed and placed in Petri dishes and supported above damp filter-paper. Moisture rapidly disappeared from the lower surfaces of the leaves, even though the weather was mainly dull. No germination occurred. Sprayed leaves on branches enclosed in polythene envelopes containing a pool of water behaved in the same way, even when cool air was bubbled through a sintered plate in the pool.

Inoculated 1 cm.^{2} leaf disks were floated, lower surface uppermost, in Petri dishes nearly full of water in an incubator with a Perspex door in front of a laboratory window. After allowing a period for equilibrium to be be reached, they were sprayed. Even then, some loss of water was observed after 2 hr. No germination was detected.

Leaves in the field were inoculated and sprayed during drizzling rain in daylight. The lower surfaces quickly dried and were resprayed repeatedly. Even during heavy rain, drying out occurred within 45 min. No germination was found.

Germination in daylight in the field is thus not only adversely affected by the light itself but also by evaporation of water droplets. Satisfactory conditions for germination will presumably only normally occur at night.

To test whether the growth of germ tubes that had started in the dark would be inhibited by light, inoculated leaf disks were floated in Petri dishes as described above and kept in the dark. Germination was estimated on a sample and the length of the longest germ tube on twenty germinated spores measured. Half the remaining disks were then exposed to light.

In one trial the average germination after 3 hr. in the dark was 7·7%. After a further $1\frac{3}{4}$ hr. it was 6·0% in the light and 18·0% in the dark. Thus at 3 hr. it was incomplete and continued in the dark, but was inhibited in the light. The average length of germ tubes was 78 μ for those continuously in the dark and 94 μ for those exposed to light for part of the time. Thus growth had continued in the light.

In another trial, after 4 hr. dark, germination continued during a further $2\frac{1}{2}$ hr. in the dark, but not in the light. Average germ tube length after 4 hr. was 72 μ and after $6\frac{1}{2}$ hr. 146 and 86 μ respectively. Thus further growth occurred in the light but less than in the dark. By examining the probit frequency distribution curves for germ tube lengths, it was deduced that germ tubes which were less than 30 μ long on exposure to light were completely inhibited from further growth. Above this length there was decreasing inhibition with increasing length and none above 110 μ.

The results indicate that germination commencing before dawn will not continue, though germ tubes may go on growing if they have reached 30 μ in length.

Germination and appressorium formation in the field

In a series of trials, leaves on a large potted plant in the open were inoculated using a damp paint brush to pick up spores and distribute them over the lower surface. At a set time or times they were lightly sprayed with water and together with the subtending branch enclosed in a polythene envelope. Spraying was done at various times from 8 a.m. to 10 p.m. and examinations, using the cellulose acetate method, were made at 10 p.m. the same day or 8 a.m., 6 p.m., 8 p.m. or 10 p.m. the next day.

Nil or very low germination was recorded by dusk for spores wetted in the morning. Considerable germination occurred between 6 and 10 p.m., but it was not usually completed in this time. Only in one case out of four was germination by 10 p.m. increased by the spores having been wet during the day. Appressorium formation was detected the following morning at 8 a.m. when wet conditions commenced at 6 p.m. and in three trials when they commenced at 10 p.m.

With one doubtful exception there was no increase of germination from continuing moist conditions through the next day, but in one trial a statistically significant increase in percentage of appressoria was found. By the evening most of the germ tubes had disintegrated. Thus if rain falls by 10 p.m. appressorium formation may be expected by the next morning and may continue during that day if wet conditions are maintained. From the laboratory observations, it is likely that a period of at least 3–4 hr. of wet conditions before dawn is necessary before appressorium formation can commence, and rain would have to fall not later than 2 a.m. If it falls later, but at least 2 hr. before dawn, and wet conditions continue after dawn, infection may perhaps occur. The possibility needs further investigation.

The period required for infection

Leaves on bushes in the field were each inoculated by transferring spores to five small water-droplets placed near the mid-rib and between the main lateral veins. Pairs of old, medium-aged, and young leaves were inoculated on each branch, which was then lightly sprayed with water and enclosed in a polythene sleeve. Different branches were inoculated at 1 p.m., 6.30 p.m. and 10 p.m. The sleeves were removed from half the branches at 8 a.m. the following day and from the remainder at 8 a.m. the day after. The leaves then rapidly dried off. During enclosure moist conditions

Table 1. *The percentage of inoculated points developing rust lesions on leaves maintained moist for various periods*

Moist period		Percentage developing rust lesions
Commenced	Terminated 8 a.m.	
Monday 1 p.m.	Tuesday	20
	Wednesday	3 } 10·9
Monday 6 p.m.	Tuesday	5
	Wednesday	18 } 11·8
Monday 10 p.m.	Tuesday	7
	Wednesday	7 } 7·0
Total	Tuesday	11·0 —
	Wednesday	8·7 —

were maintained by periodic respraying. The inoculated areas were recorded for rust lesions $7\frac{1}{2}$ weeks later and the percentages showing them are given in Table 1, which shows that maintenance of moist conditions from 10 p.m. to 8 a.m. was sufficient to produce infection and that no increase in percentage infection resulted after a further 24 hr. of moist conditions. The percentage of areas infected on old, medium-aged and young leaves was 12·7, 9·5 and 9·1 %, respectively. There was thus no evidence for young leaves being more susceptible.

The incubation period

Observations on the seasonal variations in the incubation period were made on three trees cropping well and three poorly. Inoculations were made as described above at intervals of 14–28 days on two young and two old leaves per branch.

On some occasions only pale-yellow, non-sporing areas were produced. It is noteworthy that this was most noticeable under drought conditions when the incubation

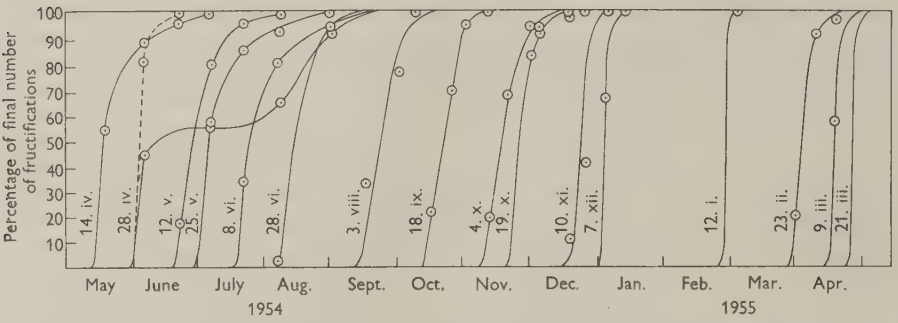

Fig. 2. The progress with time of sporing fructification development expressed as a percentage of the total number finally developing for inoculations carried out at various times during the year. The respective date of inoculation is noted on each curve.

period was also very long. Lesions resulting from inoculations on a given date did not all appear simultaneously. Therefore the number of inoculated areas with sporing lesions was expressed as a percentage of those finally developing. These were plotted against time and curves fitted by eye (Fig. 2), from which the time required for 5, 50 and 95 % to sporulate was estimated. The difference between the 5 and 50 % points varied with inoculation date from 1 to 9 days and that between the 50 and 95 % points from 1 to 32 days. The time to reach maximum spore-producing activity, roughly estimated, varied from 6 to 13 weeks and sporing was largely finished in 7–18 weeks in the absence of leaf shedding.

The effect of date of observation on the 5 and 50 % points is shown in Fig. 3. Apart from the rather anomalous very long incubation period for the inoculation of 12 January it was longest during May to September, the cooler months.

It was often possible to relate rust outbreaks in the field during these observations to specific dates of infection. Thus the rust maximum in early April could only have been induced by a rain shower on 20 February, this being the only rain that month. This incubation period, $5\frac{1}{2}$ weeks, was the same found by inoculation. The only

heavy showers previous to a strong development of young spots on 13 September were on 28 and 29 July, giving $6\frac{1}{2}$ weeks incubation. The first heavy storm of the 'Short Rains' on 8 November was followed by a strong development of spots on 12 January, a $4\frac{1}{4}$ week period. The only marked fall of rain the month before the next outbreak on 9 March was on 4–6 February, $4\frac{1}{2}$ weeks previously. The following outbreak at the end of April was preceded by the beginning of the 'Long Rains' in the third week of March, suggesting a 5-week incubation. Most of the periods deduced agree well with expectation from the inoculations.

The percentages of successful inoculations showed no statistically significant effects of age of leaf or size of crop on the trees.

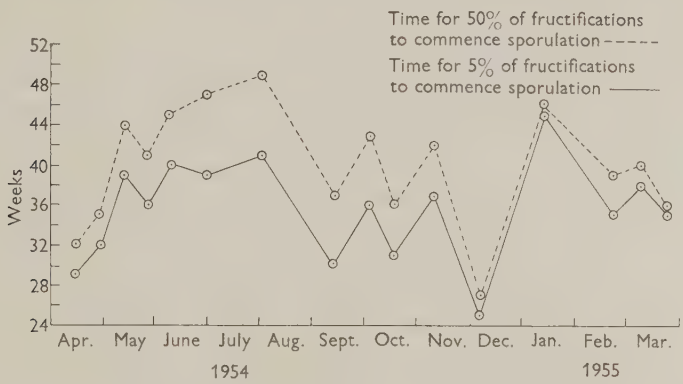

Fig. 3. The variation of incubation period with time of the year.

The relationship between temperature and incubation period was investigated. As seasonal variations of temperature in Kenya are not adequately characterized by average temperatures the mean maxima (X_1) and minima (X_2) during the incubation period were used to calculate a multiple regression equation, $Y = 90{\cdot}61 - 0{\cdot}408X_1 - 0{\cdot}440X_2$, where Y was the estimated incubation period (50 % point).

The agreement between observed and calculated periods was best from May to August. The main departure was that of the inoculation of 12 January, when the period was much longer than estimated, probably due to drought. No spore production from that of 9 February occurred, although yellow spots resulted. The other main departures were in the early 'Long Rains' and the late 'Short Rains', the periods being shorter than expected. Both were periods of heavy rains and thus may represent the opposite to the drought effect.

Incubation periods for points near the upper and lower altitude ranges of coffee planting in Kenya have been estimated from temperature data. The averages were around 33 days for Makuyu (4800 ft.) and 37 and 38 for Upper Kiambu (5700 ft.). Although the difference is not large, it may be critical in determining the number of generations possible in a given length of rainy season and hence the rate of disease build-up. In addition in cold weather in Upper Kiambu the period increases to 42 days.

The incubation periods found for Kenya are very considerably longer than those

reported from elsewhere. Ward (1882) reported 15 days for old leaves and 10–11 for young ones to produce yellow areas, sporing requiring 2–4 days more. His observations were apparently made in a laboratory at about 78° F. Mayne (1933) at Ballehonnur, Mysore, South India, also working under laboratory conditions, found 15–24-day incubation periods. Estimates calculated from the equation on p. 503 for the temperatures which applied showed that these were probably largely responsible for the shorter periods found.

In testing for varietal susceptibility and for the determination of rust races much information on incubation periods in the laboratory in the open and in a greenhouse was accumulated. The range observed was 19–63 days with race I (*sensu* D'Oliveira (1955–57)) and 26–48 days with race II. The longest periods with race I were found with the coffee variety K7, which is immune to race II.

Considerable differences between varieties were observed, up to 17 days with race II and 28 days with race I. On the whole those giving shorter periods with one race also did so with the other. However, their relative position differed. Thus Amphillo gave rather shorter periods than Harar with race I, but considerably longer ones with race II. With both varieties and with both races they were shorter than with SL34.

The high field susceptibility of Harar may in part be related to the very short incubation period shown by both rust races on this host. Another factor is the greater number of lesions which it shows from the same degree of inoculation. The reverse occurs with K7, which is susceptible to race I, but at Ruiru it was very difficult to obtain successful inoculation and the incubation period was very protracted. During the warmer seasons and in the greenhouse, inoculation was more frequently successful and the incubation period shorter. In the field at Ruiru (5400 ft.) and at the Scott Laboratories, Nairobi (5900 ft.), true K7 plants are not attacked. At lower altitudes, however, a fair infection may develop on K7, but it is never so strongly attacked in such localities as other varieties such as SL. 34 and SL. 28, which are susceptible to both races I and II.

DISCUSSION

In the recent reviews by Wellman (1957) and Razafindramamba (1958) 24 hr. is stated to be necessary for germination and Bürk's (1887) finding of a 2–2½ hr. minimum has been overlooked. The present observations are in accord with those of Bürk and the whole process of infection has been shown to be complete in 10 hr. and may possibly take less time.

In the literature young leaves are frequently said to be more easily infected than old, a statement probably mainly originating with Bürk (1887). However, he apparently carried out no inoculations to compare susceptibility and based his view on considerations as to which leaves remained wet long enough for infection to occur. The present investigations do not support his conclusions. Further, in numerous determinations of race or varietal susceptibility, young leaves were found difficult to infect, possibly largely because water tends to run off them very easily. No difficulty was found in infecting mature leaves of any age. With natural infection young lesions have been observed on leaves of all ages except those still of juvenile (glossy) appearance. This appearance is lost at between 5 and 23 (average 12·2) weeks of age (Rayner, 1951).

As the incubation period averages 5 weeks it is evident that leaves are rarely infected until fully expanded.

From the present investigations it is clear that germinations can normally occur only at night. Infection will also occur at night or possibly during the following day if the leaves remain wet. Its amount over a period of days will be related to the number of nights during which water droplets are present on the undersurfaces of the leaves. In producing these, rain is more important than dew, according to the writer's observations in the East Rift of Kenya, since the latter has been rarely observed to form on the lower leaf surfaces and only then usually shortly before dawn. The number of nights on which leaves are wet at nightfall, or in which rain falls before 10 p.m., may be taken as a lower limit of the number of occasions on which infection is likely to occur. Once wet at dusk, the leaves remain so until the following morning. An upper limit may perhaps be fixed by the number of nights rain falls before 2 a.m. For practical purposes in forecasting rust outbreaks the difference between these upper and lower limits will be small and probably of little importance. Further investigation could fix the upper limit more precisely and determine whether other factors, such as the physiological state of the plant and seasonal variation in other meteorological factors have any significant effect on the time required for infection.

A more detailed account of these investigations is deposited at the Coffee Research Station, Ruiru, Kenya and at the Commonwealth Mycological Institute, Kew, England.

The investigations described were carried out whilst I was Plant Pathologist and Physiologist at the Kenya Department of Agriculture's Coffee Research Station, Ruiru, and were completed whilst in receipt of a Colonial Development and Welfare Fund grant from the Colonial Office, London; half of the sum being contributed by the Coffee Board of Kenya. For accommodation and much encouragement I am sincerely grateful to Dr Hopkins, Director of the Commonwealth Mycological Institute, Kew. For assistance in carrying out much of the experimental work, I have to thank Miss D. Napper and Mrs W. Nicholls.

REFERENCES

Bennett, S. H. & Furmidge, C. G. L. (1956). Spray application problems: XXIV. The study of plant surfaces and spray deposits by means of transparent impressions. *Annu. Rep. Long Ashton Res. Sta. for 1955*, p. 121.

Bürk, W. (1887). Over de Koffiebladziekte en de middelen om haar te bestrijden. *Meded. PlTuin, Batavia* **4**, 6.

D'Oliveira, B. (1955–57). As ferrugens do Cafeeiro. *Rev. do Café Português.* **1** (4), 5; **2** (5), 5; **2** (6), 5; **2** (7), 9; **2** (8), 5; **4** (16), 5.

Mayne, W. W. (1933). Annual Report of the Coffee Scientific Officer, 1931–1932. *Bull. Mysore Coffee Exp. Sta.* no. 7.

Morgan, N. G. (1960). A simple suction apparatus for the uniform deposition of dry fungal spores on detached leaves. *Annu. Rep. Long Ashton Res. Sta. for 1959*, p. 126.

Rayner, R. W. (1951). *Kenya Dept. Agric. Annu. Rep. 1949*, Vol. II, p. 49.

Razafindramamba, R. (1958). Biologie de la rouille du Caféier. *Rev. Mycol., Paris*, **23**, 177.

Ward, Marshall H. (1882). Researches on the life history of *Hemileia vastatrix* the fungus of the coffee leaf disease. *J. Proc. Linn. Soc. Bot.* **19**, 299.

Wellman, F. L. (1957). *Hemileia vastatrix*—Investigaciones presentes y pasadas en la herrumbre de café y su importancia en la America Tropical. *Fedecame: Sección de Divulgación*, no. 23, 1957.

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The effect of gibberellic acid on the incidence of loose smut of wheat

By P. G. MANTLE*
Botany Department, Imperial College, London, S.W. 7

(*Received* 10 *February* 1961)

SUMMARY

Under greenhouse and field conditions gibberellic acid treatment did not significantly affect the development of loose smut mycelium within a susceptible wheat plant. No control of the disease occurred; on the contrary, the results suggested an increased fungal growth following treatment under certain greenhouse conditions.

INTRODUCTION

Gibberellic acid has been extensively used in botanical research over the last few years and many remarkable properties have been attributed to it. Farrar (1958) suggested that gibberellic acid should control embryo-infecting smuts such as *Ustilago tritici* (Pers.) Rostr. The effect of gibberellic acid on the susceptible host/parasite interaction between loose smut and wheat has been investigated.

MATERIALS AND METHODS

Gibberellic acid was obtained in both solid and 4000 p.p.m. liquid form. A concentration of 1000 p.p.m. was used in these experiments; 100 mg. of solid gibberellic acid was dissolved in 1 ml. of absolute ethanol and made up to 100 ml. with distilled water. The control was 1 % ethanol. The 4000 p.p.m. stock solution was diluted with distilled water, and the control was distilled water alone.

EXPERIMENTAL

Experiment 1

Six pots of Little Club wheat inoculated with loose smut were sown in December 1958. After 23 days' growth in the greenhouse, at approximately 65° F. under supplementary fluorescent lighting, sturdy plants had developed. Leaf blade and sheath lengths were measured and the pots divided randomly into two groups. One group was sprayed with gibberellic acid using a hand atomizer and the other was similarly sprayed with 1 % ethanol. Other inoculated seed of Little Club was soaked in solutions similar to those used for spraying, for 16 hr. at 65° F. The seeds were then sown 4 cm. deep in John Innes compost. Subsequent seedling emergence was recorded and leaf lengths measured at frequent intervals.

After 35 days it was apparent that gibberellic acid had caused excess extension of

* Now at Biochemistry Department, Imperial College, London, S.W. 7.

the third internode, bringing the crown well above its normal position at soil level and, as this internode was not strong enough to support the weight of the epicotyl, the seedlings had toppled over.

After 38 days the lodged seedlings had become more erect and the level of the soil was raised to facilitate the normal production of secondary roots from the crown.

At maturity (mid-April 1959) there was no evidence that gibberellic acid had influenced the development of the fungus within the growing-point region, or its subsequent sporulation in the ear, nor was there marked foliar response to treatment. The most marked differences between treatment and control were in the plants from soaked seed (Table 1). There was also a marked increase in flag-leaf smutting correlated with gibberellic acid treatment.

Table 1. *The effect, on the third internode and subsequent smutting, of seed and seedling treatment of Little Club wheat with gibberellic acid*

	Seedling sprayed				Seed soaked				
							No. of smutted plants		
	No. of plants	No. of smutted plants	No. of plants with flag-leaf smut	Flag-leaf smut intensity score*	No. of plants	Mean excess 3rd internode above soil level	Total	With flag-leaf smut	Flag-leaf smut intensity score*
Treated	60	14	1	3	111	0·62 cm.	20	10	18
Control	63	22	1	1	112	0·00 cm.	29	2	3

* An arbitrary estimation of the intensity of flag-leaf smutting was made by scoring 1, 2, or 3 marks for slight, moderate, or heavy flag-leaf smutting, respectively. The intensity score is the sum of the individual scores.

Experiment 2

The preceding experiment resulted in an increased incidence of flag-leaf smutting following seed soaking with gibberellic acid. The effect of an identical treatment followed by growth in the field was investigated. Alter, Bersée, Kota, and Little Club wheat varieties, inoculated with loose smut, were each divided into two groups (treated and control), each group comprising one hundred seeds except in Bersée, with fifty seeds per group. The seed was sown, after soaking in the appropriate solutions, in 6 ft. rows, fifty seeds per row, on 13 April 1959. At maturity the percentage of smutted plants was almost identical in treated and control plots, and no flag-leaf smut was observed.

Experiment 3

The seed soaking treatment of Little Club (Exp. 1) was repeated as exactly as possible a year later, also using Fylgia wheat. Seed of both varieties was soaked, and in addition previously untreated Fylgia plants were sprayed with gibberellic acid 4 weeks after sowing. Gibberellic acid treatment again resulted in abnormal third-internode extension, Fylgia responding much more than Little Club. Spraying after 4 weeks resulted in a very marked response by Fylgia. The internodes extended rapidly,

causing the plants to lodge; the etiolated ears emerging a few days earlier than in seed-treated or control plants. At maturity no flag-leaf smut was observed and there was little difference between the percentage of smutted plants within each variety (Table 2).

Table 2. *The effect, on subsequent smutting, of seed and plant treatment of Little Club and Fylgia with gibberellic acid*

Treatment	No. of plants		% smutted plants	
	Little club	Fylgia	Little club	Fylgia
Control. Seed soaked in 1 % ethanol	27	40	63	70
Seed soaked in gibberellic acid	29	38	66	66
Plants sprayed with gibberellic acid 4 weeks after sowing	—	38	—	79

DISCUSSION

Farrar (1958) claimed control of a seedling-infecting smut of oats (*Ustilago avenae* (Pers.) Rostr.) by gibberellic acid, and deduced that its use should also control embryo-infecting smuts. Unfortunately, his deductions were partly based on the conclusions of Vanderwalle (1942) that loose smut mycelium lagged behind the growing point during host development and later caught up. This has since been reinvestigated (Mantle, 1961) and the persistence of mycelium in the growing point of susceptible varieties demonstrated. Farrar also referred to Batts (1955*a*), and from differing interpretations of the word plumule, made clearer in a fuller account by Batts (1955*b*), concluded that host development could be accelerated and the fungus permanently left behind.

In the present experiments, except for abnormal third-internode extension after seed treatment, very little growth response of wheat to gibberellic acid treatment was observed in the young seedlings. It appears that gibberellic acid accentuates internode extension here only when this is normally about to take place. Thus it was not possible to induce early shooting and consequently the young ear was permeated by the fungus before gibberellic acid could induce excess internode extension, and no control of the disease occurred. On the contrary, the frequency of flag-leaf smut sporulation in plants grown from seeds soaked in gibberellic acid suggested stimulation of the fungus, though this did not occur in a repeat experiment. If the increased flag-leaf smut sporulation was associated with fungal stimulation by gibberellic acid treatment, the observations of Brian, Elson, Hemming & Radley (1954) may offer a possible explanation. They found an increase in the glucose and other soluble carbohydrate content of Victor wheat grown in a nutrient solution containing gibberellic acid. This was attributed to increased carbon assimilation mainly due to increased assimilatory area. It is possible that a more abundant soluble carbohydrate supply in gibberellic-acid-treated plants accentuated the tendency for the development and subsequent sporulation of loose smut in the flag-leaves. The tendency for even the control plants to show flag-leaf smut sporulation may have been associated, in part, with the supplementary illumination. In the field none of the tested varieties, including Little Club, produced flag smut.

Thus it is concluded that the development of loose smut mycelium within a susceptible wheat host is not affected by gibberellic acid treatment at any stage in the host's growth and that, consequently, control of the disease by this substance is not possible.

The gift of samples of gibberellic acid from Plant Protection Ltd. is gratefully acknowledged.

REFERENCES

Batts, C. C. V. (1955*a*). Infection of wheat by loose smut *Ustilago tritici* (Pers.) Rostr. *Nature, Lond.* **175**, 467.

Batts, C. C. V. (1955*b*). Observations on the infection of wheat by loose smut (*Ustilago tritici* (Pers.) Rostr.). *Trans. Brit. mycol. Soc.* **38**, 465.

Brian, P. W., Elson, G. W., Hemming, H. G. & Radley, Margaret (1954). The plant growth promoting properties of gibberellic acid: a metabolic product of the fungus *Gibberella fujikuroi*. *J. Sci. Fd Agric.* **5**, 602.

Farrar, L. L. (1958). Control of covered kernel smut (*Sphacelotheca sorghi*) of grain and black loose smut (*Ustilago avenae*) of oats with gibberellic acid in greenhouse studies. *Plant Dis. Reptr.* **42**, 1254.

Mantle, P. G. (1961). Persistence of loose smut infection within the growing point region of susceptible wheat varieties. *Trans. Brit. mycol. Soc.* In press.

Vanderwalle, R. (1942). Note sur la biologie d'*Ustilago nuda tritici* Schaf. *Bull. Inst. agron. Gembloux*, **11**, 103.

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Further observations on the reaction of wheat varieties to physiologic races of loose smut

By P. G. MANTLE*
Botany Department, Imperial College, London, S.W. 7

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SUMMARY

The reaction of forty-one wheat varieties to the three Cambridge physiologic races of *Ustilago tritici* has been determined by a combination of the embryo test and growth in the field. Wide variations in susceptibility were observed and there was no consistent relationship between the extent of mycelial distribution in the scutellum and subsequent field reaction to infection. Immunity is rare. The best differential varieties for identifying the three races are suggested.

INTRODUCTION

Until recently the reaction of wheat varieties to loose smut was said to be either susceptible or resistant, according to whether or not smutted ears were produced in the field. Popp (1951, 1959) and Batts & Jeater (1958) have shown that healthy plants may develop from embryos in which the scutellum is infected with the pathogen, and that this is due to the failure of the pathogen to infect the plumule. Thus, a positive embryo test does not necessarily indicate that smutted plants would develop, and for the classification of varietal reaction it is necessary to perform an embryo test and grow inoculated seed in the field.

Distinct physiologic races, distinguished by differential hosts, have been identified in many countries, and Batts & Jeater (1958) investigated the reaction of combinations involving fifty-seven wheat varieties and two Cambridge races of loose smut. This investigation has here been extended further.

MATERIALS AND METHODS

At anthesis, ears were inoculated with loose smut spores by the hypodermic needle or partial vacuum methods in 1956 and 1958. The three Cambridge loose smut races (Batts, 1955) were obtained from seed inoculated at the N.I.A.B. A sample, usually twenty-five seeds, was tested for embryo infection, and in many combinations the extent of fungal distribution within the scutellum was estimated on an arbitrary scale (Table 1). Usually five rows, each containing seed from one inoculated ear, were field sown and the percentage of smutted plants recorded at maturity.

* Now at Biochemistry Department, Imperial College, London, S.W. 7.

Table 1. *An arbitrary scale for estimating the extent of scutellar infection*

Very slight (v.s.)	One or two hyphae in one part of the scutellar periphery
Slight (s.)	A small distinct infection focus in one part of the scutellar periphery
Moderate (m.)	Either a number of small infection foci scattered around the scutellar periphery, or a large sector of the scutellum permeated by the fungus
Heavy (h.)	Extensive distribution of the fungus within most of the scutellum

THE REACTION OF VARIETIES

Table 2. *The field reaction and extent of embryo infection of wheat varieties to Cambridge smut races. Seed inoculated* 1958

Variety	Race	Assessment of infection*		Extent of embryo infection (cf. Table 1)	Classification†
		Embryo	Field		
Adin	C3	3	3	v.s.–s.	S
Agror	C1	4	0	v.s.–s.	E
	C2	4	4	v.s.–s.	S
	C3	3	0	v.s.–s.	E
Alfy	C1	2	0	v.s.–s.	E
Alter	C1	3	3	v.s.	S
Atle	C1	2	0	s.–m.	E
	C2	2	0	m.	E
Atson	C1	1	0	v.s.	E
Aubers	C1	0	0	0	R
Benign	C1	2	0	v.s.	E
	C2	1	1	v.s.–s.	Es
	C3	2	2	s.	S
Bersée	C1	0	0	0	R
	C3	0	0	0	R
Carstens VIII	C1	2	2	s. -m.	S
	C2	1	1	v.s.–m.	S
	C3	2	2	s.–m.	S
Ceres	C2	2	1	m.–h.	Es
Dominator	C1	2	2	v.s.	S
Hest Bignon	C1	2	0	v.s.	E
	C2	2	0	v.s.	E
	C3	3	2	s.–m.	S
Hybrid D'Avrille	C1	1	0	s.	Re
	C2	2	0	v.s.–s.	E
	C3	1	1	v.s.	S
Kota	C2	3	0	s.–m.	E
Little Club	C2	3	0	s.–m.	E
Marne Desprez	C1	2	0	s.–m.	E
Marsters 57	C1	3	1	m.	Es
	C2	3	3	m.	S
	C3	3	3	m.	S
Mesnil	C1	2	0	v.s.–m.	E
Mindum	C2	1	0	v.s.	Re
	C3	2	0	v.s.	E
Minister	C1	3	0	v.s.–s.	E
	C2	2	2	v.s.	S
	C3	2	0	v.s.	E

Continued overleaf

Table 2 (*cont.*)

Variety	Race	Assessment of infection*		Extent of embryo infection (cf. Table 1)	Classification†
		Embryo	Field		
Molinel	C1	1	0	m.	Re
	C2	2	1	m.	Es
Pilote	C1	1	0	v.s.	E
	C3	1	1	v.s.	S
Poncheau	C1	0	0	0	R
	C2	0	1	0	S
	C3	0	0	0	R
Peragis 368/20	C3	2	0	s.–m.	E
Reward	C3	3	3	m.	S
Rollo	C1	3	2	m.–h.	S
Rümkers Dickkop	C2	2	0	m.–h.	E
Tavero	C1	3	2	v.s.–s.	S
9/3/1	C1	3	0	v.s.–s.	E
	C2	2	2	v.s.–m.	S

* 1 = 1–10 %; 2 = 11–40 %; 3 = 41–70 %; 4 = 71–100 %.
† S = embryo susceptible and field susceptible.
E = embryo susceptible and field resistant.
Es = as E, but occasionally (< 5 %) S.
R = embryo immune.
Rs = as R, but very occasionally (< 1 %) S.
Re = as R, but occasionally (< 5 %) E.

Table 3. *The field reaction of wheat varieties to Cambridge smut races. Seed inoculated* 1956

Variety	Reaction of smut races*		
	C1	C2	C3
Bladette de la Garonne	S	S	†
Heurtibise	R	S	E
Ideal Bataille	E	E	S
Liberator	S	S	S
Marian Bataille	R	S	R
Miche	S	S	S
Otofte 56	E	E	S
Vaillant	R	S	†
H392/14	E	E	S
327	E	†	†
1/52	E	S	E

* Classification as in Table 2.
† Not tested.

Some of the combinations described by Batts (1955) and Batts & Jeater (1958) were tested again and most of their findings were confirmed; the discrepancies are noted below.

Atle infected with race C1 has an 'E' assessment of 2, while Batts (1955) found it was susceptible. Also a higher number of Atle embryos infected with race C2 were observed here. Benign infected with race C1 was found to be 'E' and not 'S'.

A further test on Benign inoculated with race C2 in 1959 indicated that, although generally confirmed as an 'Es' combination in 1958, most of the embryos could be infected though only about half produced smutted plants, the others being field resistant. Kota, Little Club and Rümkers Dickkop inoculated with race C2, and Peragis 268/20 inoculated with race C3 all had embryo infection, though their field resistance has been confirmed. Ceres infected with race C2 was 'Es', although Batts observed no smut in the field.

Batts & Jeater (1958) regarded Molinel as almost immune to infection by loose smut as they found only a few embryos infected with race C2. Here there was 24% embryo infection and a few smutted plants in the field. A further test showed 35% embryo infection and no smut in the field.

Although no infection of Poncheau by race C2 was observed during the embryo test, there was 4% smutting in the field. However, in a further test 15% of v.s.–m. embryo infection was correlated with smutting in the field.

DISCUSSION

'Es' combinations, such as Marsters 57, Molinel, and Hest Bignon infected with races C1, C2 and C3, respectively, are particularly interesting. Popp (1951) described a combination where the pathogen appeared to lag behind during host development and only occasionally sporulated in the ear. Some 'Es' combinations may function similarly, or possibly only a few of the infected embryos had plumular bud infection. Anatomical investigation of such combinations is necessary as this type of partial resistance may not necessarily be due to the same mechanism (Mantle, 1961*a*, *b*).

Batts & Jeater (1958) concluded that, in susceptible varieties, an embryo test gives a reliable indication of the extent of field smutting. However, where the assessment of susceptibility is low, there may sometimes be a higher proportion of infected embryos of which only a few will produce smutted plants. This emphasizes the extremely variable nature of wheat-variety/smut-race interactions. In some combinations a consistent failure to infect the plumular bud of the embryo obviously imparts field resistance, while in others there are exceptions to this limitation of scutellar infection. Genetical variations in host and parasite may be a contributory feature, and where there is a delicate balance between susceptibility and resistance, environmental conditions during embryo development and subsequent early growth may also have an effect (Mantle, 1961*b*).

Hybrid D'Avrille was only very slightly susceptible (3%) to race C3, while Marsters 57 was very susceptible (63%) under the same conditions of inoculation and subsequent seed development. This illustrates an apparently real variation in susceptibility, which applies both to 'E' and 'S' types, depending only on the variety/race combinations, and the assessment of susceptibility is probably a fairly reliable measure of reaction under good inoculation conditions.

There was no consistent relationship between the intensity of embryo infection, i.e. the amount of mycelium present, and subsequent reaction to infection. Heavy smutting in the field may result from very slight embryo infection, and field resistance may be associated with moderate or heavy scutellar infection. This confirms previous

observations by Batts & Jeater (1958), but Popp (1951) considered that susceptible plants may escape infection if the embryos were lightly invaded with mycelium.

Batts & Jeater (1958) commented on the infrequent occurrence of immunity, and this has been confirmed. 'S' and 'E' combinations were equally frequent while the other types were much less frequent. Combinations with all three Cambridge races may produce any of the reactions. Field resistance was often associated with embryo infection; the inability of the fungus to reach the embryo being unusual. This prevalence of embryo infection in field-resistant varieties is a significant feature and, ideally, in the selection of parental varieties for the breeding of loose smut resistance, it would be advisable to choose those with an immunity rather than simple field resistance.

Combining these results with those of Batts & Jeater (1958) it is remarkable that only one of the varieties tested, Dominator, is susceptible only to race C 1, though this may only apply when it is winter-sown (Mantle, 1961*b*). It is, therefore, the best differential for this race.

Race C 2 is best identified by Bersée, which is immune to races C 1 and C 3. Other differential varieties for this race are Heurtibise, 1/52 and Agror.

Varieties for identifying race C 3 are H 392/14, Ideal Bataille, and Otofte 56.

This classification of varietal reaction does not take into account any seedling abnormalities (Mantle, 1961*a*), and it is possible that, in a seedling investigation of these combinations, unusual reactions to infection would be observed.

I wish to express my gratitude to the late Dr C. C. V. Batts for his active interest in this work, and to Dr D. Doling for supplying the field smutting data of seed inoculated in 1956.

REFERENCES

Batts, C. C. V. (1955). Loose smut (*Ustilago tritici* (Pers.) Rostr.) of wheat: physiologic specialization and reaction of varieties in England. *Ann. appl. Biol.* **43**, 533.

Batts, C. C. V. & Jeater, Ann (1958). The reaction of wheat varieties to loose smut as determined by embryo, seedling, and adult plant tests. *Ann. appl. Biol.* **46**, 23.

Mantle, P. G. (1961*a*). Further observations on an abnormal reaction of wheat to loose smut. *Trans. Brit. mycol. Soc.* In press.

Mantle, P. G. (1961*b*). The effect of vernalisation on loose smut infected embryos of field resistant wheat varieties, and their subsequent development. *Ann. appl. Biol.* In press.

Popp, W. (1951). Infection in seeds and seedlings of wheat and barley in relation to development of loose smut. *Phytopathology*, **41**, 261.

Popp, W. (1959). A new approach to the embryo test for predicting loose smut of wheat in adult plants. *Phytopathology*, **49**, 75.

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Observations on the use of predacious fungi for the control of *Heterodera* spp.

By A. F. HAMS and G. D. WILKIN
Research Department, Boots Pure Drug Co. Ltd., Nottingham

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SUMMARY

Laboratory and pot experiments by various workers up to 1956 suggested that the effects of eelworm attack on certain crops could be partly overcome by inoculating eelworm-infested soil with the mycelium of predacious fungi.

The present paper demonstrates some of the factors operative in soil which affect the 'predacity' of some of these fungi.

The results obtained strongly suggest that under laboratory conditions and in pot cultures congenial conditions for growth in soil of the predacious fungi can be produced but that, in the field, only sparse growth of these fungi can be obtained, insufficient to affect eelworm attacks.

The use of farmyard or green manure has consistently given better crops in eelworm-infested soils irrespective of whether predacious fungi were naturally present or added.

INTRODUCTION

The ability of certain soil-inhabiting fungi to capture and parasitize nematodes has been known for some years (Zopf, 1888) and knowledge of this biological phenomenon has greatly increased since then.

The work of Dreschler (1941) in America from 1933 until the present day, Deschiens (1939) in France and Duddington (1957) with small-scale pot trials in England from 1940 onwards, suggested that it might be possible to reduce the activities of nematodes attacking cultivated crops.

Species of predacious fungi, *Dactylaria thaumasia* and *Arthrobotrys robusta*, were used (Brook & Duddington, 1953) as single or mixed agar preparations. These were inoculated into manure which was later used in field trials on potatoes at Nocton and Dunston Fens in Lincolnshire. Larger amounts of mycelium of *Dactylaria thaumasia*, *Arthrobotrys robusta* and *Trichothecium cystosporium* were produced later by deep-culture methods (Brit. Pats. 1958).

These trials as a whole showed some indication of reduction in the severity of eelworm attack, as reflected by increased yields, but the results were erratic and difficult to reproduce, and it became clear that further information on the behaviour of predacious fungi in soil was necessary.

It appeared that the fungi would need to reduce eelworm numbers under conditions which also favoured the release or movement of eelworms through the soil. As little information on the conditions favouring the predacious fungi was available, the

laboratory work was undertaken in an attempt to establish the effect of soil environment on both eelworms and fungi, while field trials were undertaken to test improved predacious fungus material.

The following paper describes these attempts to solve some of the problems of practical eelworm control by biological methods.

LABORATORY METHODS

Spore production

In submerged culture. Arthrobotrys robusta grew and spored well in submerged culture in a whey medium of the following composition: whey powder 2·8%; lactose 3·4%; corn steep liquor 0·38%; potassium dihydrogen phosphate 0·4%; water to make 100%. The pH of the medium was adjusted to 6·8–7·0.

Reduction in the nitrogen and an increase in the electrolyte level was necessary to induce sporulation of *Dactylaria candida* and *Trichothecium cystosporium*. The spores differed from the normal reproductive conidia in being produced directly from the vegetative mycelium. The cells produced by *Dactylaria candida* under these conditions appeared to originate in the same way as sticky knobs and then to be set free in the culture fluid. This would suggest that the sticky knobs of this organism have evolved from spores.

On an inert substratum. An inoculum of the predacious fungus to be used was grown under submerged conditions and the vegetative mycelium added to four times its bulk of the basic whey medium already described. This inoculated medium was then added to vermiculite at approximately 2 ml./g. and incubated for 14–21 days at 26–28° C.

The solid was then vacuum- or air-dried leaving a mass of vermiculite containing spores of the predacious fungus. Normally the spores produced under these conditions were typical conidia characteristic of the species. *Cylindrocarpon radicicola* produced thick-walled resting cells from the mycelium.

The fungus was grown out from such preparations on agar plates containing 20 μg./ml. of aureomycin and 5 μg./ml. of penicillin to keep down the bacterial contamination.

Method of estimating potency of dried preparations

The nematicidal value of the dried preparations was assayed by the following pot method.

The dried fungus material, with bran as a nutrient for the fungus, was added to weighed amounts of field soil known to be highly infested with cereal root eelworm cysts. The whole was well mixed and divided equally amongst several (usually four) 6 in. pots. Pots containing untreated soil acted as controls.

Germinated oat seeds were planted in this mixture, six plants to each pot. The plants were then grown under controlled light and temperature (four 80 W. fluorescent tubes, 1 ft. from the plants at 20° C.) and after 3 weeks' growth, a number of the control plants were harvested at 7-day intervals and the nematodes in the root cortex counted. When a suitably high number—around 400–600 per g. of root—was found in the untreated plants, the whole experiment was stopped and the roots from each treat-

ment were preserved in 2% formalin for a later examination. The method used to estimate larval infection was to take the formalized roots and to boil in lacto-phenol containing 0·1% basic fuchsin for 5 min. After this time the whole root was mounted in lacto-phenol between two $3\frac{1}{2} \times 2\frac{1}{2}$ in. glass plates and the larvae in the cortex were counted with the aid of a binocular dissecting microscope.

The results of one such test are given in Table 1. Each figure represents the average count obtained from six separate roots taken from different pots.

The figures given by treatments 1, 5 and 6 are not significantly different.

Table 1. *Effect of preparations of predacious fungi in the laboratory*

Treatment	Larval count per root	Larval count per g. of root
(1) Control: no addition	345	750
(2) As (1) with 0·5% bran added	140	185
(3) As (2) with 0·1% dried *D. candida*	80	115
(4) As (2) with 0·1% dried *A. robusta*	80	120
(5) As (1) with dried *D. candida*	320	670
(6) As (1) with dried *A. robusta*	290	650

Method of assessment of fungal activity

To follow the growth of selected fungi in soil a respirometric method based on oxygen uptake of the fungus was used.

Pattingham soil (moderate cereal root eelworm infestation) was kept in a sterilizing oven at 110° C. for 16 hr. and checked for sterility. Dried material of *Trichothecium flagrans and Cylindrocarpon radicicola* was prepared as described above.

The soil was re-sterilized in Petri dishes in 22 g. amounts and the fungus added in 6 ml. of water or nutrient medium and uniformly distributed. The inoculated soil was then incubated in a humidity chamber at 20–22° C. for 3 weeks.

To test the growth of the added fungus in the soil approximately 2·0 g. aliquots were placed in Warburg cups to each of which was added 1·5 ml. of 0·1% phosphate buffer containing 1% glucose at the required pH. The replicate Warburg cups were shaken at 26° C. at 100 strokes per minute and the oxygen uptake of the sample recorded. The contents of the cups were then carefully washed on to tared filter-papers, dried for 30 min. at 60° C. in a vacuum oven and weighed. The oxygen uptake was calculated as μl. of oxygen taken up per gram dry weight of soil per hour and this gave a comparative measure of the respiratory activity of different samples. Sterilized soil samples without added fungus gave no significant oxygen uptake figures. The results obtained using this method to determine the effect of various factors on the growth of *C. radicicola* are given in Tables 2–4.

Effect of various factors on growth of Cylindrocarpon radicicola

Effect of pH. The effect of pH of the soil was tested by adjusting the pH of the phosphate buffer by mixing K_2HPO_4 with KH_2PO_4. The results obtained after 8 days' incubation using soil or vermiculite containing a nutrient medium are given in Table 2, which shows that this pH range had little effect on the oxygen uptake of the organism.

Effect of moisture. The optimal moisture level for activity of *C. radicicola* was assessed

Table 2. *Effect of pH on oxygen uptake of* Cylindrocarpon radicicola *(μl. oxygen/g./hr.)*

pH	Soil	Vermiculite
5·0	36·0	90·0
6·0	40·0	96·0
7·0	39·0	110·0
8·0	36·0	108·0

Table 3. *Effect of soil moisture in Petri dishes*

Volume of water added (1 ml.)	Oxygen uptake (μl./g./hr.)
1·0	Soil practically dry
2·0	12
3·0	26
4·0	30
5·0	36
6·0	43
7·0	35
	(partially waterlogged)
8·0, 9·0	Waterlogged—fungus grew as a surface growth on water-logged soil

Table 4. *The effect of fungus seeding level and the effect of nutrient fluid compared with water*

	Treatment		Days incubation	Oxygen uptake (μl. oxygen/g./hr.)
(1) (*a*)	Nutrient fluid; basic seeding	×0	6	30
(*b*)		×2	6	35
(*c*)		×4	6	60
(*d*)		×8	6	65
(2) (*e*)	Water; basic seeding	×0	6	13
(*f*)		×2	6	15
(*g*)		×4	6	45
(*h*)		×8	6	50
(3) (*a*)	Nutrient as (1)		12	40
(*b*)			12	50
(*c*)			12	35
(*d*)			12	23
(4) (*e*)	Water as (2)		12	12
(*f*)			12	15
(*g*)			12	10
(*h*)			12	10
(5) (*a*)	Nutrient as (1)		20	30
(*b*)			20	30
(*c*)			20	25
(*d*)			20	15
(6) (*e*)	Water as (2)		20	<5
(*f*)			20	<5
(*g*)			20	<5
(*h*)			20	<5

by taking 22 g. lots of sterile soil in Petri dishes and adding increasing amounts of water, then inoculating with dried vermiculite material of the fungus and determining the oxygen uptake after 8 days. The results, given in Table 3, suggest that fungus metabolism is highest when the soil is wet but not saturated.

Effect of agricultural fertilizers. Agricultural fertilizers added to soil included dried blood, ammonium sulphate, hoof and horn, bone meal, Compure N* and Compure K*, at the recommended levels. In no instance did the fertilizer increase the persistence of the fungus over that produced by the addition of water only.

Growth cycle in soil. Table 4 shows the effect of initial level of inoculation of fungus into the soil, together with the persistence of growth in the soil when either 6 ml. water or the same volume of a nutrient solution was added. The nutrient solution used was the whey medium previously described.

From the results obtained it is seen that the fungus persists longer in soil when a nutrient solution is present and the increasing seeding rates result in a faster disappearance of fungus. This is presumably due to exhaustion of soil nutrient resulting from the more rapid proliferation of the fungus when used at a higher inoculum level.

It would appear therefore that abnormal soil conditions, e.g. very high and very low pH or high water content reduce fungal growth. It is also shown that fertilizers, essential to healthy plants, are not likely to enhance the fungal growth. The lack of improved growth of the fungus under these conditions shows that the protection from eelworm attack it might afford the plants is unlikely to be increased by the use of economic agricultural fertilizer levels.

FIELD AND POT TRIALS

Dactylaria thaumasia was first applied as minced-up agar cultures mostly provided by Duddington. Large amounts of mycelial tissue were produced by submerged culture fermentation processes and used in 1956, but this material was very bulky and difficult to distribute evenly and was unstable except under refrigeration.

Later, Wilkin produced, still by deep-culture methods, resistant spore forms of some fungi (see page 515), which could be freeze-dried and stored as a dry powder; this was used in 1957. The freeze-drying of large quantities of material was difficult, and in 1958–59 the 'spores' were dispersed either in vermiculite or kieselguhr, which was then filter-and vacuum-dried.

1957 *trials*

In 1956 a large pot trial including *D. thaumasia*, *Trichothecium cystosporium* and *A. robusta* applied to potatoes, tomatoes and peas had shown that the latter improved the growth of peas giving nine times the yield of the untreated pots. The effect of the other fungi on tomatoes and potatoes was less well defined.

In the 1957 trials, 'resistant' spore forms of *Arthrobotrys robusta* and *Dactylaria candida* were used in comparison with filter-dried mycelium of *A. robusta*. Sufficient was applied, based on germinating spore counts, to give 40×10^6 viable spores per square yard of plot area. Supplementary trials on peas were made in which half, twice and four times the original rates for both fungi were included. In these two smaller trials the only organic dressing used was farmyard manure.

* Proprietary liquid fertilizers.

The randomized plot trials were conducted in fields chosen for their high eelworm infestation levels. The potato and pea trials were laid out and sown on 27 March and 4 April, respectively. Besides the use of organic additives with the fungus in the trial plots, two other methods of application were tried, one to soil and the other as a seed dressing. The only effective treatments and results are given in Table 5 for both peas and potatoes.

Table 5. *Treatment and crop fields*

	Mean yield	
	Potatoes (lb.)	Peas (g.)
(1) Chopped cabbage + *A. robusta* (spores)	3·9*	195
(2) *A. robusta* (mycelium)	4·3*	286
(3) *A. robusta* (alone)	3·7*	267
(4) *D. candida* (spores)	3·9*	232
(5) Soil untreated	2·7	129

* Results significant at $P = 0{\cdot}05$.

Bran or farmyard manure combined with the fungi as in treatments 1–4 including half, twice and four times the rate of seeding (using F.Y.M. only) failed to give statistically significant yield increases. Soil applications and seed dressing also gave no significant effect using the spore or mycelial forms of both fungi.

The organic materials were used at the rate of 2 tons of dry matter per acre.

An analysis of variance carried out on the weights for potatoes showed that the increase necessary for significance was 0·96 lb. where $P = 0{\cdot}05$; treatments 1–4 were the only ones to reach such a level.

Statistical analysis of the main pea trial showed that no significant differences existed. However, treatments 1–4 again showed a greater yield than the others. One striking feature common to all three organic groups, viz. F.Y.M., bran and chopped cabbage, with the exception of treatment 4, was the failure of the spore form of the fungi to produce any improvement.

Neither the soil application nor the seed-dressing methods appeared to be of any real value. Throughout the whole trial the mycelial form of *A. robusta* produced the greatest effect, but never enough to be a statistically significant improvement over the organic additive alone. Chopped cabbage and *A. robusta* mycelium was the most satisfactory combination.

1958 *trials*

The fungus preparations (*Dactylaria candida*, *Trichothecium cystosporium* and *Phialophora heteroderae*) used in these trials were all 'spore' forms absorbed on to vermiculite or kieselguhr.

A fertilizer formulated to contain equivalent amounts of the various chemical elements found in the fungus preparations was applied to some plots to meet the criticism that any apparently beneficial fungus effect might be due solely to the nutritional effect of the mycelium.

Another innovation was the introduction of a reputed eelworm-cyst-invading fungus —*Phialophora heteroderae*—both in the main trials and at one site on fallow land, where viable cysts counts only were used as the basis for activity assessment.

The third innovation was the use in this trial of F.Y.M. inoculated 5 weeks before use with *Dactylaria candida* and *Trichothecium cystosporium*. This treatment was included in an attempt to reproduce the beneficial effect obtained with similar material in 1955.

Two crops were used, oats (on two sites) and potatoes (on one site). Farmyard manure applied at 15 tons/acre was the only organic material used. All the fungus preparations were applied to the top 8 in. of soil at 0·025 % by weight.

Broadly speaking it can be said that the use of F.Y.M. gave some improvement, but that no further increases in height or yield followed the use of the predacious fungi. The numerical results have been omitted as there were no striking differences between treatments. In this respect they were basically a repetition of those obtained in 1957.

A trial was also carried out to determine the effect on the viable cyst population exerted by *Phialophora heteroderae*. The cyst counts given in Table 6 were made by the Entomology Department at Shardlow (East Midland Regional H.Q., N.A.A.S.) Treatments were as follows:

(1) Farmyard manure.
(2) Farmyard manure plus *P. heteroderae* (70 g./sq.yd.)
(3) Untreated soil.
(4) Untreated soil plus *P. heteroderae* (70 g./sq.yd.)

Table 6. *Viable cyst counts from samples taken on the date shown*

Treatment	29. iv. 58		4. vi. 58		3. vii. 58		6. viii. 58		6. ix. 58	
	A	B	A	B	A	B	A	B	A	B
1	40	86	29	52	48	148	22	31	43	122
2	—	—	46	142	30	68	34	93	44	24
3	—	—	38	127	42	87	26	55	29	56
4	—	—	48	169	34	53	45	32	29	55

Where A = % viable cysts recovered from 100 g. of samples of soil. B = no. eggs per g. of soil.

Table 6 shows that there was little variation in percentage viable cysts. The majority of cysts extracted were normal in appearance and not blackened by *P. heteroderae*, the usual symptom of parasitism.

In the trials where yield was assessed there is little evidence of improvements produced by the fungi alone. Such increases as were demonstrated can be attributed to the introduction of F.Y.M.

1959 *trials*

A primary trial was set up using a mixture consisting of four parts Pattingham soil to one part Hackthorn soil (moderate and very high cereal root eelworm cyst infestation levels, respectively). Oats were used as a test plant.

For treatments 5 and 6 the liquid medium was that normally used for the deep culture of *C. radicicola* and was applied whenever the other pots were watered.

This trial was set up on 21 May and seedling samples were withdrawn on 8 and 17 June and used for estimating the larval invasion counts given in Table 7. As the larval counts in the primary trial were unexpectedly low, a subsidiary trial was

arranged using Hackthorn soil only and treatments 2, 4, 10, 11 and 12. Larval invasion counts made on this trial are given in Table 8.

The seeds from the subsidiary trial were sown on two dates separated by 9 days. This theoretically gave the fungus time to proliferate prior to seedling root growth. Samples for invasion counts were likewise taken on 2 separate days.

Table 7. *Larval invasion counts after treatments*

	Sample 8 June	Sample 17 June
(1) Soil + 0·5 % (w/w) bran	130	250
(2) Soil + 0·5 % (w/w) bran + 0·2 % *Cylindrocarpon radicicola*	250	215
(3) Soil + 0·5 % (w/w) soya flour	100	250
(4) Soil + 0·5 % (w/w) soya flour + 0·2 % *C. radicicola*	150	390
(5) Soil + liquid-culture medium (as required)	380	540
(6) Soil + liquid-culture medium (as required) + 0·2 % *C. radicicola*	130	210
(7) Soil + 2·5 % (w/w) green manure	470	170
(8) Soil + 2·5 % (w/w) green manure + 0·2 % *C. radicicola*	90	150
(9) Soil + 2·5 % (w/w) F.Y.M.	260	560
(10) Soil + 2·5 % (w/w) F.Y.M.	370	320
(11) Soil + 0·2 % (w/w) *C. radicicola*	140	160
(12) Untreated soil	260	360

Table 8. *Subsidiary trial*

	Larval invasion figures (larvae/g. root)	
Treatment	1st sample	2nd sample
2	190	230
4	100	No counts taken
8	105	150
10	100	70
11	400(?)	70
12	80	25

There is a slight rise in count from the first to second date, but no indication of a high degree of control of eelworm entry by the fungus.

The above results suggest that treatment 8 gives some stimulation to the fungus, but the figures obtained are not significantly different from treatment 11 (no organic additive) by 17 June.

None of the treatments reduced the invasion count below that of untreated soil and there is no evidence that the organic additives or the added fungus had any effect on the eelworm entry.

DISCUSSION

The pot trials carried out in the laboratory showed that conditions in the soil could be made sufficiently congenial for the introduced predacious fungus to reduce considerably the number of eelworm larvae entering the host roots. Samples of soil from the field when used in laboratory pot trials gave higher invasion figures than were obtained in the field trials and the introduction of predacious fungi had a much greater effect in pots under controlled conditions than in the field.

The results obtained strongly suggest that energetic trapping of larvae by the fungus is only carried out when there is a high degree of mycelial proliferation, and respiro-

metric determinations show that even with stimulants such as bran the peak growth occurs about 12–15 days after 'seeding'. This period of 12–15 days is sufficient time for adequate root development of the seedlings, acceptable to the larvae, to take place but unless larval emergence from the cysts is also at a peak at this time, the fungus will not be capable of reducing their numbers to any great extent. Under normal field conditions where the fungus is existing at a minimum subsistence level, growth would be expected to be insufficient to have a marked effect on the eelworm fauna.

Of the organic materials introduced to stimulate the fungus, it can be said that both F.Y.M. and chopped cabbage have at times produced increased yields, but it is difficult to prove that superimposition of fungus on these treatments produced a significant improvement in the field.

The failure to achieve in the main field trials any measurable improvement in crop yield by introducing fungus may be due to the fact that the 'peak' of fungal growth did not coincide with the 'peak' of larval emergence from the cysts.

As the fungal treatments must be applied, from a practical angle, at the time of seed sowing, unless the weather conditions are such that the above coincidence occurs within a relatively short time, the chances of the fungus entrapping sufficient larvae to protect the crop are small.

The success of the laboratory pot trials implies that the experimental conditions were congenial for both the fungus and larvae, and that the 'peaks' did coincide in these trials.

The apparent failure of *Phialophora heteroderae*, the cyst-invading fungus, to achieve any control is possibly correlated with soil conditions, the adequate control of which the previous field trials have shown to be virtually impossible. Success would have been encouraging, as the use of this fungus would have obviated the necessity for the attempted provision of soil conditions favourable for fungal growth and eelworm cyst hatching—prime requisites for the economically effective behaviour of the eelworm trapping fungi.

It would thus appear (*a*) that either the experiments described have not solved the problem of the correct use of predacious fungi for the field control of eelworms, or (*b*) that the results obtained suggest that this type of biological control is ineffective on a practical scale.

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REFERENCES

B.P. 857,160. (1958). Improvements in the propagation of fungi.
B.P. 857,161. (1958). Improvements in the propagation of fungi.
Brook, M. & Duddington, C. L. (1953). Personal communication.
Deschiens, R. (1939). Considerations relatives à la destruction des larves de nematodes parasites par des Hyphomycetes predateurs. *Bull. Soc. Path. Exot.* **32**, 459.
Dreschler, C. (1941). Predacious fungi. *Biol. Rev.* **16**, 265.
Duddington, C. L. (1957). *The friendly fungi.* 188 pp. London. Faber and Faber.
Zopf, W. (1888). Zur Kentniss der Infections-Krankheiten niederer Thiere und Pflanzen. *Nova Acta Acad. Leop. Carol.* **52**, 314.

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Cherry leaf-roll virus

By R. CROPLEY
East Malling Research Station, near Maidstone, Kent

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SUMMARY

A virus was transmitted consistently by sap inoculation from cherry trees with leaf-roll disease to herbaceous indicator plants, from tobacco to *Prunus avium* and from *P. avium* to *P. pennsylvanica.*

Herbaceous host reactions, physical properties and particle size and shape of the virus were similar to those of arabis mosaic, raspberry ringspot and tomato black-ring viruses. Plant protection and gel-diffusion serological tests indicated that cherry leaf-roll virus is distinct from arabis mosaic, raspberry ringspot, tomato black-ring, tobacco ringspot and tomato ringspot viruses.

INTRODUCTION

Most viruses that infect sweet cherry trees in England cause no conspicuous symptoms in many varieties of *P. avium* but decrease the growth and cropping of trees in commercial orchards. Such viruses were present in more than 90 % of the apparently healthy cherry trees tested in England, probably resulting in an annual reduction in potential crop of at least 20 % (Posnette & Cropley, 1960). Cherry leaf-roll virus is less prevalent but its effect on most cherry varieties is so severe that the trees rapidly become unproductive (Posnette & Cropley, 1955; Posnette, 1956).

SYMPTOMS AND DISTRIBUTION

Infected trees (*P. avium*) are delayed in leafing and flowering in the spring. In summer the margins of the leaves roll upwards and in some varieties turn purplish (Pl. 1, fig. 1). Trees decline in vigour and gum often exudes from splits in the bark; individual branches are killed and eventually the entire tree dies. The disease has been found in ten orchards in Kent, one in Berkshire and one in Worcestershire; in some of these, only one or two trees are infected, while in others many trees are dying, contiguous with young trees planted to fill gaps caused by earlier deaths, probably from leaf-roll disease.

The virus nature of this disease was established by graft-transmission tests to young trees of the varieties Early Rivers, Bing, Geante de Hedelfingen and Schrecken Bigarreau (Posnette, 1956). Florence, Van and Lambert were later also shown to be sensitive. Two distinct syndromes developed on *P. avium* F 12/1 plants infected from diseased trees in different orchards. In one experiment, F 12/1 plants were infected in July by bud-grafts from trees in five different orchards. Inoculations with buds

from diseased trees in three orchards caused leaf-roll symptoms on the F 12/1 indicator plants in the year after budding, and gum-filled swellings developed on the stems in the following year. Subsequent growth was much reduced but there was little die-back. Similar inoculations from infected trees in two other orchards caused necrosis and gumming of F 12/1 stems around the inoculation buds 9 months after budding; leaves above the inoculation buds became cupped and red, and the stems above the point of inoculation died in the following year. This apparently eliminated the virus infection, as symptomless shoots grew from below the inoculation buds, and scions taken from these shoots 43 months after infection did not induce the disease when grafted to healthy plants of F 12/1.

Virus transmission from infected trees

Leaves from infected cherry trees were macerated in four times their own weight of 0·05 M phosphate buffer (pH 7·8) or 0·01 M sodium diethyldithiocarbamate, 'Celite' abrasive was added and the sap rubbed on to the leaves of *Nicotiana tabacum* L. var. White Burley, *Chenopodium amaranticolor* Coste and Reyn, *Petunia hybrida* Vilm. and *Cucumis sativus* L. var. Bucher's Disease Resister plants. Tobacco and *Chenopodium* were very susceptible to infection by the virus from cherry sap, and in later tests cucumber and petunia were omitted when isolating it from cherry trees. In such tests a virus was consistently transmitted from diseased trees, but not from normal trees in the same orchards. The virus was readily transmitted from tobacco to other herbaceous plants (Table 1).

Properties in vitro

Sap extracted from freshly harvested tobacco leaves and clarified by centrifugation at 8000 *g* for 10 min. was used in the experiments. Infectivity was tested by adding a small quantity of 'Celite' and inoculating the leaves of White Burley tobacco plants.

Dilution end-point. Sap from systemically infected leaves, or inoculated leaves harvested 8–12 days after inoculation, was infective at 1/100, but rarely at 1/1000: preparations that were infective at 1/1000 were not at 1/10,000.

Thermal inactivation. Undiluted sap, with a dilution end-point of between 1/100 and 1/1000, was infective after heating for 10 min. at 52° C. but not at 55° C.

Longevity. Crude sap stored at 20° C. was infective after 5 days but not after 10 days. Sap stored at −15° C. was infective after 30 days.

Precipitation by chemical agents. No loss of infectivity was detected when equal volumes of sap and saturated ammonium sulphate solution were stored at 18° C. for 45 min., centrifuged for 15 min. at 8000 *g* and the pellet resuspended in water. Precipitates formed by 50% acetone or 50% ethanol were only slightly infective.

Particle size. Infective tobacco sap was clarified by freezing at −15° C. for 10 days and centrifuging at 8000 *g* for 15 min. The clarified sap was given two cycles of high- and low-speed centrifugation (90,000 *g* for 150 min. and 8000 *g* for 10 min.) and the final pellet suspended in 1/20 of the original volume. Mr H. L. Nixon (Rothamsted Experimental Station) kindly made electron micrographs of these preparations. Particles fixed with formalin had an average diameter of 32 mμ, and shadows cast by these particles were similar to those cast by particles of arabis mosaic, raspberry ringspot and tomato black-ring viruses (Harrison & Nixon, 1960).

Table 1. *Herbaceous host plants of cherry leaf-roll virus*

Host plant	Symptoms	
	Inoculated leaves	Systemically infected leaves
Nicotiana tabacum L. var. White Burley	Necrotic spots and rings after 1½–4 days (Pl. 1, fig. 2)	Yellow spots, rings and lines, sometimes becoming necrotic; subsequent leaves symptomless (Pl. 1, fig. 3)
N. glutinosa L.	Necrotic spots after 3–5 days	Yellow spots and mottle
N. rustica L.	Chlorotic spots and rings after 6 days	Chlorotic rings
Chenopodium amaranticolor Coste and Reyn	Chlorotic and necrotic spots after 3–5 days	Mottle, distortion and necrosis; growth stunted
C. quinoa L.	Necrotic spots and areas after 3–5 days	Mottle, distortion and necrosis; young plants sometimes killed
Phaseolus vulgaris L. (French bean) var. Prince	Necrotic spots after 4 days	Brown spots and rings, vein necrosis; during winter death of growing tip
Cucumis sativus L. (Cucumber) var. Bucher's Diesease Resister	Summer: chlorotic spots on cotyledons. Winter: necrotic spots, wilting and death of cotyledons	Summer: yellow spots, rings and lines on a few leaves. Winter: mottle and distortion, growth very stunted
Petunia hybrida Vilm.	None	Summer: usually none. Winter: sometimes a mild mottle
Vinca rosea L.	None	None (the virus was transmitted from these leaves to tobacco)
Plantago major L.	Few small brown etched spots on upper surface after 10 days	Necrotic etched spots and areas, coalescing to form rings. Sometimes vein necrosis and leaf distortion
Solanum nigrum L.	Chlorotic spots and rings after 6 days	Chlorotic rings
Fragaria × *ananassa* Dutch. (Strawberry) var. Cambridge Favourite	None	Yellow spots and blotches on a few leaves

Plant protection tests

Protection tests were made on symptomless leaves of 'recovered' tobacco plants previously infected with cherry leaf-roll virus. One half of each leaf was re-inoculated with cherry leaf-roll virus, and the other half with one of the following viruses: arabis mosaic (raspberry yellow dwarf) virus (Harrison, 1958*a*), tomato black-ring virus (supplied by Dr Kenneth Smith), a beet ringspot strain (Harrison, 1957) and a lettuce ringspot strain (Smith & Short, 1959) of tomato black-ring virus, raspberry ringspot virus (Harrison, 1958*b*), and American strains of tomato ringspot and tobacco ringspot viruses (supplied by Dr Kenneth Smith). Cherry leaf-roll virus did not prevent the developments of lesions produced by any of the challenge viruses. In reciprocal tests, none of these viruses protected against infection with cherry leaf-roll virus.

Serological tests

An antiserum was prepared by injecting a rabbit intravenously with virus precipitated by ammonium sulphate from 10 ml. of frozen and clarified sap from infected tobacco leaves. Five injections were given at 4-day intervals, and the rabbit bled 6 days after the final injection. Double diffusion tests were made in a 3 mm. thick agar gel of 0·7 % 'Ionagar' No. 2, 0·9 % sodium chloride and 0·02 % sodium azide. Holes 7 mm. in diameter and 5 mm. apart were cut in the gel with a cork borer, and filled with either diluted antiserum or sap from systemically infected *Chenopodium* leaves. The titre of infected *Chenopodium* sap was variable, but was usually between 1:8 and 1:16. Most tests were made with undiluted *Chenopodium* sap and antiserum diluted 1:8. In such tests, all leaf-roll isolates formed single precipitation lines with leaf-roll antiserum, but not with raspberry ringspot or arabis mosaic antisera. Tomato black-ring virus (three strains), arabis mosaic virus (four strains), tomato ringspot virus and tobacco ringspot virus formed no precipitation lines with cherry leaf-roll antiserum (Pl. 2, fig. 4). Cadman (1960) found that cherry leaf-roll virus was precipitated by an anti-serum he had prepared against a virus isolated from a cherry tree in Holland with 'rozetziekte' (Pfaeltzer, 1959). This antiserum (kindly supplied by Dr Cadman) precipitated with all of the English leaf-roll isolates, and the Dutch virus precipitated with the English leaf-roll antiserum.

Sap extracted from expanding buds and young leaves of infected cherry trees was antigenic with leaf-roll antiserum during April and early May. Undiluted sap, or sap diluted 1:2 with 0·05 M phosphate buffer (pH 7·8) or 0·01 M sodium diethyl-dithiocarbamate, formed single precipitation lines with leaf-roll antiserum but none with arabis mosaic or raspberry ringspot antisera. Sap from healthy cherry trees and from cherry trees infected with arabis mosaic, raspberry ringspot, necrotic ringspot, little cherry, ring mottle and rusty mottle viruses was not antigenic with leaf roll, arabis mosaic or raspberry ringspot antisera (Cropley, 1960). No specific serological activity could be detected in cherry sap during June, September or October, although the virus was transmitted from cherry leaves to tobacco and *Chenopodium* plants during these months. During the autumn cherry sap was so mucilaginous that leaves had to be macerated in three times their own weight of buffer or sodium diethyl-dithiocarbamate solutions to obtain a liquid that could be squeezed through muslin. With such preparations precipitation was not specific to the antiserum, but was dependent on the type of leaf from which the sap was extracted. Sap from green leaves of healthy cherry trees formed no precipitation lines with leaf-roll, arabis mosaic and raspberry ringspot antisera or with normal serum; sap from leaves that were red in colour due to infection with leaf-roll or little cherry virus, or to constriction of the trunk by a tie, formed single precipitation lines with these antisera and with normal serum.

Sap transmission to Prunus *seedlings*

Young seedlings of *Prunus avium*, *P. pennsylvanica* and *P. persica* were inoculated when 10–20 cm. tall by dusting their leaves with carborundum powder (400-mesh) and rubbing with virus preparations in either tobacco or cherry sap. *P. pennsylvanica*

seedlings were the most susceptible and no infection occurred in peach (Table 2), although peach could be infected by graft transmission from cherry.

Symptoms appeared 2–5 weeks after inoculation; clearly defined chlorotic areas and occasional red rings and lines developed on systemically infected leaves of *P. avium* seedlings, on two of which the leaves became rolled 7 weeks after inoculation. Small chlorotic spots, rings and lines developed on systemically infected leaves of *P. pennsylvanica* seedlings (Pl. 2, fig. 5); some of these leaves became necrotic but later-formed leaves were symptomless. Gum exuded from the stem of one plant, which died 6 weeks after inoculation. Leaf-roll virus was transmitted to tobacco or *Chenopodium* from all plants that developed symptoms.

Table 2. *Transmission of cherry leaf-roll virus to* Prunus *seedlings*

Inoculum	*P. avium*	*P. pennsylvanica*	*P. persica*
Tobacco sap diluted 1:4 with 0·01 M sodium diethyl-dithiocarbamate	3/10*	3/10	0/5
Tobacco sap diluted 1:4 with 0·05 M sodium phosphate	4/19	3/9	0/20
Cherry sap diluted 1:4 with 0·01 M sodium diethyl-dithiocarbamate	0/5	3/6	—

* Plants infected/plants inoculated.

DISCUSSION

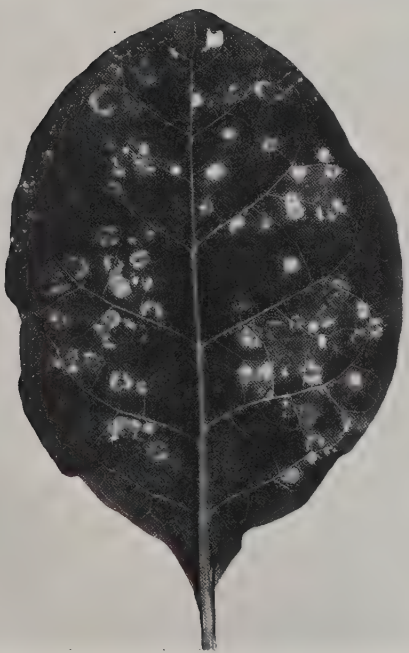
The economic importance of cherry leaf-roll virus is difficult to assess. There is no doubt that it has caused decline and death of many trees in several orchards, and field observations suggest that many others are affected. Several of the symptoms induced by this virus are similar to those caused by fungal root pathogens and by branch cankers caused by *Pseudomonas mors-prunorum*, and some of the outbreaks that have been investigated were in the past wrongly attributed to these causes.

The localized distribution of the disease in orchards suggests that the virus may be soil-borne, but attempts to recover the virus from the roots of tobacco seedlings grown in pots of soil from around diseased trees have so far given negative results. The particle size, physical properties and several herbaceous host reactions are similar to those of the soil-borne arabis mosaic, raspberry ringspot and tomato black-ring viruses, but plant protection and gel-diffusion serological tests indicated no relationship. Such tests would probably not show a distant relationship, however, such as that existing between arabis mosaic virus and viruses isolated from grapevine plants with fanleaf diseases (Cadman, Dias & Harrison, 1960).

The reactions of some herbaceous hosts distinguish cherry leaf-roll from other similar viruses. It does not induce enations on the leaves of cucumber plants, as does tomato black-ring virus; unlike raspberry ringspot virus it infects *Chenopodium amaranticolor* systemically; and it produces primary lesions on tobacco leaves more rapidly than does arabis mosaic virus, and later-formed leaves are symptomless.

Among the viruses known to infect *Prunus* spp., cherry leaf-roll appears to be unique in its ability to maintain antigenicity in cherry sap. This may be merely a reflexion of high virus concentration, however, as indicated by the large number of primary lesions that develop on tobacco leaves inoculated with infected cherry sap.


1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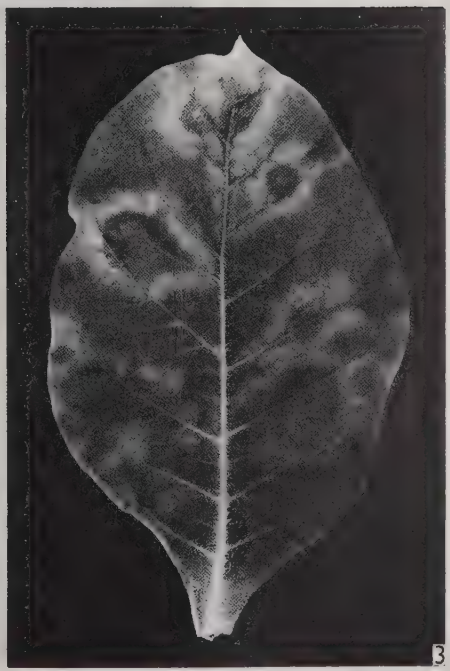
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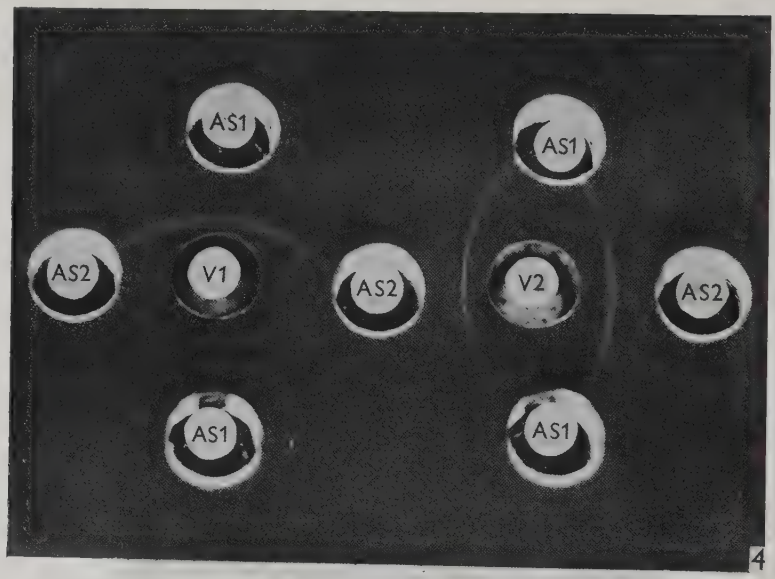

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REFERENCES

Cadman, C. H. (1960). In *Annu. Rep. Scot. Hort. Res. Inst. for* 1959–60, p. 49.

Cadman, C. H., Dias, H. F. & Harrison, B. D. (1960). Sap-transmissible viruses associated with diseases of grape vines in Europe and North America. *Nature, Lond.* **187**, 577.

Cropley, R. (1960). Serological detection of a virus in cherry trees with a leaf roll disease. *Nature, Lond.* **188**, 875.

Harrison, B. D. (1957). Studies on the host range properties and mode of transmission of beet ringspot virus. *Ann. appl. Biol.* **45**, 462.

Harrison, B. D. (1958*a*). Raspberry yellow dwarf, a soil-borne virus. *Ann. appl. Biol.* **46**, 221.

Harrison, B. D. (1958*b*). Further studies on raspberry ringspot and tomato black ring, soil-borne viruses that affect raspberry. *Ann. appl. Biol.* **46**, 571.

Harrison, B. D. & Nixon, H. L. (1960). Purification and electron microscopy of three soil-borne plant viruses. *Virology*, **12**, 104.

Pfaeltzer, H. F. (1959). Onderzoekingen over de rozetziekte van de kes. *Tijdschr. PlZiekt.* **65**, 5.

Posnette, A. F. (1956). The leaf roll virus disease of sweet cherry. *Tijdschr. PlZiekt.* **62**, 49.

Posnette, A. F. & Cropley, R. (1955). Leaf roll: a virus disease of cherry. *Rep. E. Malling Res. Sta. for* 1954, p. 126.

Posnette, A. F. & Cropley, R. (1960). Virus diseases of cherry trees in England. III. Crop reduction caused by some viruses. *Rep. E. Malling Res. Sta. for* 1959, p. 92.

Smith, K. M. & Short, Margaret E. (1959). Lettuce ringspot: a soil-borne virus disease. *Plant Pathology*, **8**, 54.

EXPLANATION OF PLATES

Photographs taken by Mr E. Yoxall Jones

Plate 1

Fig. 1. Three-year-old cherry trees infected with cherry leaf-roll virus. Healthy trees on left.

Fig. 2. Lesions on leaf of White Burley tobacco inoculated with cherry leaf-roll virus.

Fig. 3. White Burley tobacco leaf systemically infected with cherry leaf-roll virus.

Plate 2

Fig. 4. Gel diffusion serological plate. AS 1, arabis mosaic antiserum. V 1, arabis mosaic virus. AS 2, cherry leaf-roll antiserum. V 2, cherry leaf-roll virus.

Fig. 5. Symptoms of cherry leaf-roll virus in systemically infected *Prunus pennsylvanica* leaves.

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Viruses causing rasp-leaf and similar diseases of sweet cherry

By R. CROPLEY
East Malling Research Station, Near Maidstone, Kent

(*Received* 21 *February* 1961)

SUMMARY

Trees with rasp-leaf symptoms have been found in seventeen orchards in England. In some orchards only a few trees are infected, while in others the disease is prevalent.

Transmission tests from diseased trees indicate that raspberry ringspot virus is the cause of cherry rasp-leaf disease in most English orchards, but arabis mosaic virus can cause similar symptoms.

INTRODUCTION

Rasp-leaf disease was first described from North America (Bodine & Newton, 1942); narrow leaves with enations on their undersurfaces were characteristic. Diseases characterized by similar leaf enations on sweet cherry have been described from Switzerland as Pfeffingerkrankheit (Blumer & Geering, 1950), Holland as Eckelraderziekte (Mulder, 1951), Germany (Kotte, 1951), Hungary (Németh & Kegler, 1960) and England (Posnette, 1951). Until the relationships of the causal viruses have been established it seemed premature to coin numerous names to describe similar syndromes, and the name rasp-leaf was used for the disease occurring in Britain.

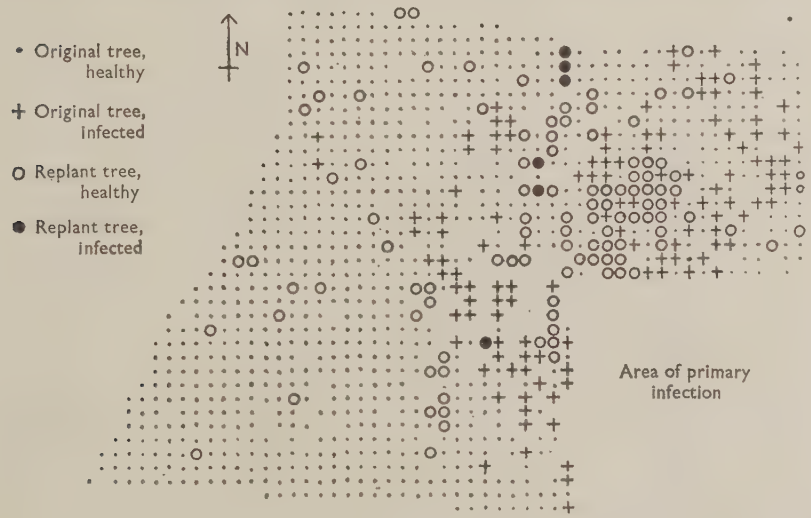
Kunze (1958) transmitted a virus 'of the tobacco ringspot group' from trees with Pfeffingerkrankheit in Germany: this virus, and viruses isolated from diseased trees in Switzerland, have been identified by Cadman (1960) as strains of raspberry ringspot virus (Harrison, 1958*b*). Two viruses have been isolated from Dutch trees with Eckelraderziekte or rozetziekte (Pfaeltzer, 1959): one is related to cherry leaf-roll (Cropley, 1961), and the other to raspberry ringspot virus (Cadman, 1961). Soil transmission to cherry seedlings of the virus or viruses causing rozetziekte has been reported (Evenhuis, Mulder & Pfaeltzer, 1959), and a virus isolated from trees with rozetziekte has been transmitted by sap inoculation from tobacco and cucumber to cherry (Pfaeltzer, 1960).

SYMPTOMS AND DISTRIBUTION IN ENGLAND

The leaves of affected trees are usually reduced in size, narrow and tough, with abnormally coarse serrations (Plate fig. 1). During spring and early summer the young leaves have clearly defined chlorotic areas ('oil flecks'). Enations on the undersurfaces of the leaves may be few or numerous, large and 'cockscomb' in shape or small and confined to areas of the leaf close to the mid-rib. Both types of enations occur in the same tree, either simultaneously or at different stages of infection. During

the first few years after infection, sensitive varieties such as Bing, Geante de Hedelfingen, Governor Wood and Knight's Early Black often produce numerous enations; as the trees decline in vigour, enations usually become smaller and less numerous. Less sensitive varieties, including Roundel, Florence and the rootstock clone F 12/1 develop only a few small enations, while the variety Early Rivers rarely produces any. Young trees of the sensitive variety Bing, inoculated by bud-grafts in July, usually show conspicuous symptoms in the following year.

The disease has been found in seventeen orchards in England, but no survey has been attempted and it is probably widely distributed. Three of these orchards were variety collections, in which the disease was confined to three or four varieties, with no evidence of spread, suggesting that the use of infected graftwood was responsible



Text-fig. 1. Distribution of cherry trees with rasp leaf in a Berkshire orchard.

for infection. In endemic outbreaks, rasp-leaf disease was often confined to one area of the orchard. In some orchards only three or four trees were infected while in others the disease was prevalent. In one Berkshire orchard of 40-year-old trees, the grower first noticed rasp-leaf symptoms in 1952 in the south-east corner of the orchard (Plate fig. 1). The number of diseased trees increased annually, and by 1957 a block of 5 acres in the original area of infection was grubbed because the trees were uneconomic. By 1959, 120 of 486 trees in the eastern part of the orchard were showing leaf symptoms, but only two of 504 trees in the western part. In recent years young trees were planted to fill gaps caused by the removal of unproductive trees, and of 116 replants under 10 years old, six (estimated to be 8 years old) were showing symptoms in 1959.

Virus transmission from infected trees

Herbaceous plants were inoculated with sap from trees with rasp-leaf symptoms by the method described for the transmission of cherry leaf-roll virus (Cropley, 1961). *Chenopodium amaranticolor* and *Petunia hybrida* were usually more susceptible to infection with the viruses associated with rasp leaf than were tobacco and cucumber. Viruses transmitted from infected trees in eight orchards produced local lesions on the inoculated leaves of *Chenopodium amaranticolor*, but did not cause systemic infection; these isolates rarely induced symptoms on the inoculated leaves of *Petunia hybrida*, but systemically infected leaves were mottled, with chlorotic ring and line patterns.

Table 1. *Virus transmission from cherry trees with rasp-leaf disease*

Orchard	Locality	Variety	Symptoms	Virus isolated
1	Worcestershire	Early Rivers	Narrow tough leaves	AMV
2	Kent	Chesley Tartarian	Mild rasp leaf	AMV
3	Kent	Napoleon	Mild rasp leaf	AMV
3	Kent	Napoleon	None	—
3	Kent	Early Rivers	None	—
4	Kent	Napoleon	Rasp leaf	RRSV
5	Dorset	Swiss Black	Rasp leaf	RRSV
6	Kent	Turkey Heart	Rasp leaf	RRSV
7	Kent	Roundel	Rasp leaf	RRSV
8	Kent	Unknown	Rasp leaf	RRSV
9	Kent	Governor Wood	Rasp leaf	RRSV
9	Kent	Governor Wood	Rasp leaf	RRSV
9	Kent	Unknown	Rasp leaf	RRSV
9	Kent	Governor Wood	None	—
9	Kent	Early Rivers	None	—
10	Kent	Florence	Rasp leaf	RRSV
10	Kent	Florence	Rasp leaf	RRSV
10	Kent	Florence	Rasp leaf	RRSV
10	Kent	Roundel	Rasp leaf	RRSV
10	Kent	Florence	None	—
10	Kent	Florence	None	—
10	Kent	Roundel	None	—
10	Kent	Roundel	None	—
11	Berkshire	Napoleon	Rasp leaf	RRSV
11	Berkshire	Frogmore	Rasp leaf	RRSV
11	Berkshire	Bradbourne Black	Rasp leaf	RRSV
11	Berkshire	Amber	Rasp leaf	RRSV
11	Berkshire	Black Cluster	Rasp leaf	RRSV
11	Berkshire	Amber	Tatter leaf	—
11	Berkshire	Early Rivers	Tatter leaf	—
11	Berkshire	Amber	None	—
11	Berkshire	Frogmore	None	—

Isolates from three other orchards infected *Chenopodium* systemically, causing distortion and mottling in the young leaves. The symptoms produced and the physical properties of the first group of viruses were similar to those described by Harrison (1958*b*) for raspberry ringspot virus, while the second group resembled arabis mosaic (raspberry yellow dwarf) virus (Harrison, 1958*a*).

Double-diffusion serological tests were made in agar gel; the three isolates that infected *Chenopodium* systemically were tested by using undiluted *Chenopodium* sap

as the antigen, and petunia sap was used for the other isolates. All isolates were tested against raspberry ringspot virus antiserum (kindly supplied by Dr C. H. Cadman) and an antiserum prepared to arabis mosaic virus isolated from strawberry at East Malling. All the virus isolates that produced only local lesions in *C. amaranticolor* gave specific precipitation lines with raspberry ringspot antiserum, and the three isolates that infected *Chenopodium* systemically reacted with arabis mosaic antiserum (Table 1). Another raspberry ringspot virus antiserum, prepared from the virus isolated from a rasp-leaf cherry tree in orchard 6 (see Table 1), reacted with all of the raspberry ringspot virus isolates in gel diffusion tests, but not with the three arabis mosaic virus isolates. Gel diffusion tests with arabis mosaic were reliable throughout the year when *Chenopodium* sap was used as the antigen. Results were reliable during the winter months with raspberry ringspot virus in petunia sap, but during the summer months virus titre was sometimes too low for the formation of a visible precipitation line.

DISCUSSION

The transmission of raspberry ringspot virus from diseased trees in eight orchards, and failure to transmit the virus from normal trees in the same orchards, strongly suggest that raspberry ringspot virus causes rasp-leaf symptoms in cherry trees. The transmission of raspberry ringspot virus from trees with similar symptoms in Germany and Switzerland suggests that Pfeffingerkrankheit and the rasp-leaf disease of many English orchards are identical, but the relationship between these diseases and the American rasp-leaf disease is not yet known. One distinction is that raspberry ringspot virus moves more rapidly through the tree than the American virus, which often remains localized for several years (Milbrath, 1954). The relationship of these diseases to Eckelraderziekt (rozetziekte) in Holland remains obscure, since both cherry leaf-roll virus and raspberry ringspot virus have been transmitted from Dutch trees (Cadman, 1961). In two English orchards some trees in each orchard have leaf-roll symptoms from which cherry leaf-roll virus has been isolated, and other trees have rasp-leaf symptoms from which raspberry ringspot virus has been isolated. It seems likely that some of the Dutch trees contained both viruses, but whether dual infection is necessary for the production of Eckelraderziekte symptoms is unknown.

The transmission of arabis mosaic virus, but not raspberry ringspot virus, from three trees with rasp-leaf-like symptoms in England may be fortuitous. However, graft transmission from two of these trees to the variety Bing induced smaller enations and less leaf narrowing than those produced by graft transmissions from trees with raspberry ringspot virus (Plate figs. 2, 3), and it seems plausible that arabis mosaic virus and raspberry ringspot virus, which have similar physical properties and produce similar symptoms in several herbaceous host plants, might cause somewhat similar symptoms in cherry.

Both arabis mosaic and raspberry ringspot are soil-borne viruses, and soil transmission is probably the most important means of infection in cherry orchards. However, reciprocal transmission of rusty mottle and ring mottle viruses between adjacent cherry trees, indicative of natural root grafting, has been reported (Posnette & Cropley, 1961) and virus spread by this means may be important in old orchards.

Cadman (personal communication) has found that some raspberry varieties are immune to raspberry ringspot and others to arabis mosaic virus. The use of immune rootstocks for cherry would enable the present range of scion varieties to escape infection with soil-borne viruses. Tests of a wide range of potential cherry rootstocks for immunity are now in progress.

REFERENCES

Blumer, S. & Geering, J. (1950). Das Kirschbaumsterben im Baselland (Pfeffingerkrankheit). *Phytopath. Z.* **16**, 300.

Bodine, E. W. & Newton, J. H. (1942). The rasp leaf of cherry. *Phytopathology*, **32**, 333.

Cadman, C. H. (1960). Studies on the relationship between soil-borne viruses of the ringspot type occurring in Britain and Continental Europe. *Virology*, **11**, 653.

Cadman, C. H. (1961). Viruses associated with diseases of the rasp-leaf type in sweet cherry. *Rep. of Proc. of Fourth Symposium on virus diseases of fruit trees in Europe*, 1960. (In the Press.)

Cropley, R. (1961). Cherry leaf-roll virus. *Ann. appl. Biol.* **49**, 524.

Evenhuis, H. H., Mulder, D. & Pfaeltzer, H. J. (1959). De overdracht van de rozetziekte, een virusziekte van de kers. *Tijdschr. PlZiekt.* **65**, 122.

Harrison, B. D. (1958*a*). Raspberry yellow dwarf, a soil-borne virus. *Ann. appl. Biol.* **46**, 221.

Harrison, B. D. (1958*b*). Further studies on raspberry ringspot and tomato black ring, soil-borne viruses that affect raspberry. *Ann. appl. Biol.* **46**, 571.

Kotte, W. (1951). Die Schmalblattrigkeit der Süsskirsche (Pfeffingerkrankheit) auch in Deutschland. *Phytopath. Z.* **17**, 468.

Kunze, L. (1958). Ein Virus der Tabak-ringflecken-gruppe von Süsskirsche. *Phytopath. Z.* **31**, 279.

Milbrath, J. A. (1954). The Eckelrader disease or Pfeffingerkrankheit. *Plant Dis. Reptr.* **38**, 258.

Mulder, D. (1951). De Eckelrader virusziekte van zoete kersen. *Meded. Dir. Tuinb.* **14**, 217.

Németh, M. & Kegler, H. (1960). Untersuchungen über das Vorkommen von Kirschenvirosen in Ungarn. *NachrBl. dtsch. PflSchDienst, Berl.* **14**, 110.

Pfaeltzer, H. J. (1959). Onderzoekingen over der rozetziekte van de kers. *Tijdschr. PlZiekt.* **65**, 5.

Pfaeltzer, H. J. (1960). 'Rozetziekte' of cherry, transmitted from herbaceous hosts to cherry seedlings. *Tijdschr. PlZiekt.* **66**, 24.

Posnette, A. F. (1951). Virus diseases of sweet cherry. *Rep. E. Malling Res. Sta. for* 1950, p. 209.

Posnette, A. F. & Cropley, R. (1961). European rusty mottle disease of sweet cherry. *Rep. E. Malling Res. Sta. for* 1960, p. 85.

EXPLANATION OF PLATE

Photographs taken by Mr E. Yoxall Jones

Fig. 1. Cherry variety Governor Wood with rasp-leaf symptoms, from which raspberry ringspot virus was transmitted.

Fig. 2. Rasp-leaf symptoms on leaf of Bing cherry infected by a graft from a tree with raspberry ringspot virus.

Fig. 3. Small enations on leaf of Bing cherry infected by a graft from a tree with arabis mosaic virus.

R. CROPLEY

(*Facing p.* 534)

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The toxicity of mixtures of zinc and copper sulphates to rainbow trout (*Salmo gairdnerii* Richardson)

By R. LLOYD
Water Pollution Research Laboratory, Stevenage, Herts.

(*Received* 24 *February* 1961)

SUMMARY

The toxicity to rainbow trout of a mixture of zinc and copper sulphates in relatively low concentrations can be calculated from the toxicities of the individual metals by assuming that they exert a similar joint action. Higher concentrations of the mixture in soft water exhibit a synergistic action.

INTRODUCTION

Waste waters which are discharged to rivers often contain more than one poisonous substance, but there have been relatively few investigations of the toxicity of mixtures of poisons to fish. Where two poisons are present in the same system, their combined effect can be exerted in one of four ways; thus, the toxicity may be predictable from the separate toxicities of the individual substances (either similar or independent joint action as defined by Bliss, 1939), or may be greater or less than predicted (synergism or antagonism).

An example of synergistic action widely quoted in the literature on fish toxicology is that of the toxicity of a mixture of zinc and copper salts. Bandt (1946) gives results of experiments on the toxicity of heavy metals to fish and states that mixtures of copper and zinc are synergistic; experimental data published by Doudoroff (1952) strongly suggest that these metals in combination are synergistic in soft water. However, it is not possible to predict from the published data the extent of the increase in toxicity resulting from the synergistic action and the present paper describes two experiments designed to give more complete data on the combined toxicities of these two metals.

METHODS

Two series of tests were made, one in a hard unchlorinated borehole water (total hardness 320 mg./l. as $CaCO_3$) and the other in an artificial soft water (total hardness 15–20 mg./l. as $CaCO_3$) prepared by diluting the borehole water with demineralized water and adding 15 mg./l. sodium chloride to the final solution (Lloyd, 1960). Both zinc and copper were added to the solutions as sulphates; the ratio of zinc to copper (as mg./l.) used in the solutions containing both metals was 6:1. Tests were made in 40 l. volumes of solution, aerated with a controlled mixture of air and carbon dioxide to give a concentration of about 8·0 mg./l. free carbon dioxide in solution; ten rainbow trout (about 3 in. long) were used in each test. The sequence of tests, and the distribution of the fish between the test aquaria, were randomized in both the hard- and

soft-water series. Rainbow trout to be used in the hard-water series were acclimatized to room temperature (15·5–17·0° C.) and free carbon dioxide concentration for 1 day before the test; those used in the soft-water series had previously been living in hard water and were acclimatized to the dilution water and room temperature (17·0–18·0° C.) for 5 days, with acclimatization to free carbon dioxide concentration on the last day. Survival times were measured from the time of immersion to the time when all respiratory movement ceased; distributions of survival times were logarithmic normal and median periods of survival were estimated graphically (Bliss, 1937). Fish which survived for more than 24 hr. were transferred to fresh solutions daily and were fed on alternate days.

RESULTS

(*a*) *Hard water*

Since zinc and copper salts differ in the concentration of metal required to kill rainbow trout in a given period of time, the following method was used to relate median periods of survival to concentrations of the metals, either when present singly

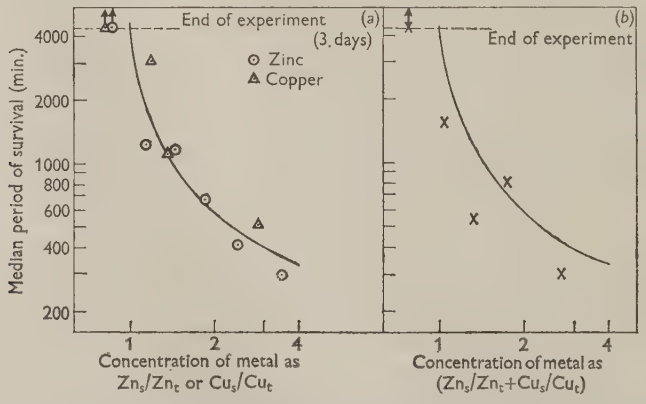

Fig. 1. Toxicity of zinc and copper sulphates in a hard water (total hardness 320 mg./l. as $CaCo_3$). (*a*) Zinc and copper singly. (*b*) Mixture of zinc and copper in ratio 6:1.

or in combination. Concentrations of copper and zinc in hard water which gave median periods of survival equal to the duration of the experiment (3 days) were determined graphically by probit analysis from the experimental data; under the conditions of the experiments in hard water, these concentrations were 3·5 mg./l. Zn and 1·1 mg./l. Cu, which will be termed Zn_t and Cu_t, respectively. The concentrations of zinc and copper used in the tests can now be expressed as proportions of these concentrations, that is, as Zn_s/Zn_t and Cu_s/Cu_t, where Zn_s and Cu_s are the concentrations of zinc and copper in the test solutions. Fig. 1*a* gives the results for the two metals when tested singly and shows the relation between the logarithm of median period of survival and the logarithm of Zn_s/Zn_t and Cu_s/Cu_t; the curve, drawn by eye, appears to fit both sets of experimental data, which suggests that both metal salts exert their toxic action in a similar way.

Results of tests in solutions containing both zinc and copper are given in Fig. 1*b* with the logarithm of $(Zn_s/Zn_t + Cu_s/Cu_t)$ as abscissa. The ratio of Zn_s/Zn_t to Cu_s/Cu_t in this experiment was about 2:1. The curve in Fig. 1*b* is the same as that which relates period of survival to concentration of the individual metals in Fig. 1*a*, and since it fits the experimental points obtained for the mixture quite well, it is reasonable to assume that, for these experimental conditions, both metal salts exert their toxic action in a similar way when present together in solution. Thus, in such a mixture, a given quantity of one metal could be substituted for an equally toxic quantity of the other without altering the toxicity of the mixture, and the results are an example of similar joint action as defined by Bliss (1939).

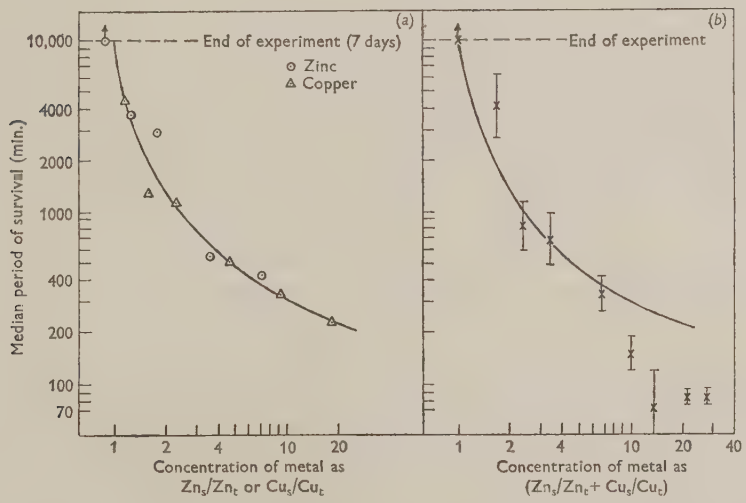

Fig. 2. Toxicity of zinc and copper sulphates in a soft water (total hardness 15–20 mg./l. as $CaCO_3$). (*a*) Zinc and copper singly. (*b*) Mixture of zinc and copper in ratio 6:1.

(*b*) *Soft water*

The data from a similar series of experiments made in soft water are shown in Fig. 2*a*; under these conditions the value of Zn_t was 0·56 mg./l., and Cu_t was 0·044 mg./l. for a 7-day median period of survival. Again, a single curve describes the relation between the logarithm of the median period of survival and the logarithm of concentration of both zinc and copper where these are expressed as Zn_s/Zn_t and Cu_s/Cu_t. Experimental points for the relation between the logarithm of the median period of survival and the logarithm of the concentration of a mixture of zinc and copper, expressed as $(Zn_s/Zn_t + Cu_s/Cu_t)$ together with the standard deviations of the individual survival times from the median, are shown in Fig. 2*b*; the ratio of Zn_s/Zn_t to Cu_s/Cu_t was about 1:2 in these experiments. The curve drawn in Fig. 2*b* is that for the individual metals in Fig. 2*a* and although it fits the data for low concentrations of zinc and copper reasonably well, which suggests that similar joint action occurs here,

the survival times of rainbow trout in high concentrations of the mixture are distinctly shorter than would be predicted from the curve, suggesting that synergism occurs in this region.

DISCUSSION

These results indicate that the toxicity of a mixture containing relatively low concentrations of zinc and copper in either hard or soft water can be calculated from the toxicities of the individual metals by assuming that they exert a similar joint action. It is of some interest that synergism occurs with higher concentrations of the mixture in soft water, since this confirms the findings of previous authors. The reason for this departure from similar joint action is not known, but may depend upon the relative proportions of zinc, copper, and calcium. This possibility could not be investigated in the tests with hard water, since concentrations of metal greater than those used were precipitated as the basic salt in this dilution water. For practical purposes, however, the concentrations of zinc and copper which could be present in a river without causing harm to a resident population of rainbow trout will be lower than those found to be toxic within a few days in these experiments and it is reasonable to assume that the combined toxicity of these two metals in such concentrations will conform to similar joint action, and that no further factor to allow for synergism need be introduced.

Mr H. T. Mann assisted with the experimental work. This paper is published by permission of the Department of Scientific and Industrial Research.

REFERENCES

Bandt, H. J. (1946). Ueber verstärkte Schadwirkungen auf Fische, insbesondere über erhöhte Giftwirkung durch Kombination von Abwassergiften. *Beitr. WasserChem.* **1**, 15.

Bliss, C. I. (1937). The calculation of the time mortality curve. *Ann. appl. Biol.* **24**, 815.

Bliss, C. I. (1939). The toxicity of poisons applied jointly. *Ann. appl. Biol.* **26**, 585.

Doudoroff, P. (1952). Some recent developments in the study of toxic industrial wastes. *Proc. Fourth Pacific Northwest Industrial Waste Conf., State Coll. of Washington, Pullman, Wash.*, p. 21.

Lloyd, R. (1960). The toxicity of zinc sulphate to rainbow trout. *Ann. appl. Biol.* **48**, 84.

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Field observations on the control of blossom beetles (*Meligethes aeneus* F.) and cabbage-seed weevils (*Ceuthorhynchus assimilis* Payk.) on mustard-seed crops in East Anglia

By A. L. WINFIELD
National Agricultural Advisory Service, Brooklands Avenue, Cambridge

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SUMMARY

Field trials on the control of blossom beetles (*Meligethes aeneus* F.), and cabbage-seed weevils (*Ceuthorhynchus assimilis* Payk.) were carried out in East Anglia during 1958 and 1959, and twenty-six commercial crops of Trowse mustard (*Brassica juncea* Coss) were surveyed during the summer of 1959. The development of a Trowse mustard plant, and damage by adult and larval blossom beetles are described. Methods for assessing the damage done by both blossom beetles and seed weevils are discussed, and a preliminary attempt has been made to differentiate the effects of the two pests on the yields of seed.

It has been confirmed that DDT is unsatisfactory for the control of seed weevils; two carefully timed applications of dieldrin will probably give an economic control of both blossom beetles and seed weevils. Spraying usually gave increased yields of seed, sufficient to cover the cost of treatment, but a single spray was not persistent enough to give a good control of the damage by adult and larval blossom beetles, and at the same time to control the damage by seed weevil larvae. A single spray applied during May, when the population of adult blossom beetles was rising, did not prevent later adults or larvae from causing damage. A single spray applied shortly before flowering began allowed the early immigrant adults to cause damage but gave some control of the larvae. The infestation of pods by seed weevil larvae was best controlled by a spray during the early-flowering period. Because of the risk of harming bees and other beneficial insects it is unlikely that a spray during the blossom period could at present be recommended as a routine for all mustard crops.

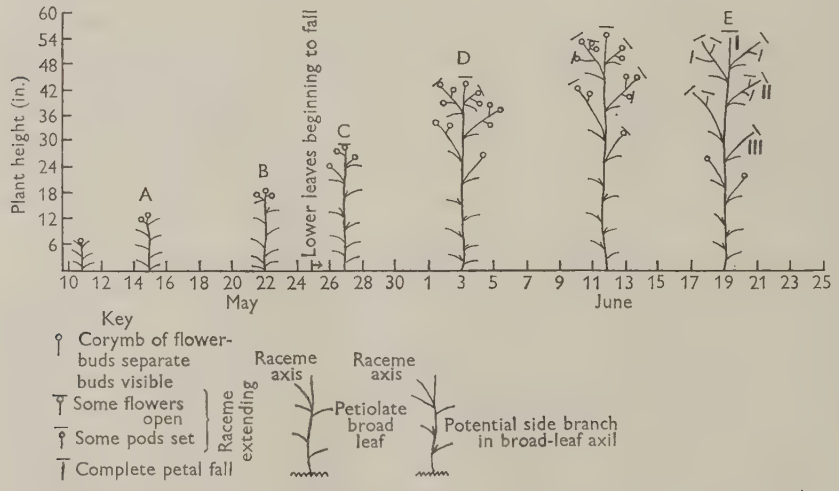
INTRODUCTION

Potter & Perkins (1946), and Jary & Carpenter (1948) found in England that DDT dusts gave a satisfactory control of blossom beetles (*Meligethes aeneus* F.) on various brassica crops. Field and laboratory studies on the biology and control of blossom beetles and cabbage-seed weevils (*Ceuthorhynchus assimilis* Payk.) attacking winter and spring rape crops have been made on the continent by many workers, notably Holz (1948), Frey (1950), Herrström (1951), Godan (1952), Bonnemaison (1951, 1952), Hascoët (1953), Bauers (1954), Risbec (1952*a*, *b*), Bouron (1955) and Ankersmit (1956). Preliminary observations on the two insects attacking mustard-seed crops have been

briefly reported by Winfield & Gough (1959). The work was done mainly on the brown-seeded Trowse mustard (*Brassica juncea* Coss), and the choice of insecticides and the timing of the treatments have been based on the conclusions of the continental authors. In 1958 several insecticides were tested, and further field trials on spray timing were carried out in 1959. Also in 1959 a small survey was carried out on several commercial crops of Trowse mustard in Norfolk, the Isle of Ely, and the Holland division of Lincolnshire.

THE MUSTARD PLANT AND BLOSSOM BEETLE DAMAGE

A series of diagrams representing the development of a Trowse mustard plant is shown in Text-fig. 1, and the letters allotted to each stage are used with the insect population curves later in the paper.



Text-fig. 1. Development of Trowse mustard (*B. juncea* Coss), showing raceme subsampling. The main stages are A 'green-bud'; B 'yellow-bud'; C 'first open flowers'; D 'first pods setting'; E 'complete petal fall'. The date at which each stage occurs is influenced by sowing date, site and season. This series was drawn from plants grown on light mineral soil at Trumpington, Cambridge, sown on 18 March 1959. Raceme subsampling at stage E: 1958 main racemes from positions I and II (UM); 1959 and 1960, main racemes from positions I, II and III (UML).

Each fully grown plant (E) has a main stem and usually four to seven side-branches arising from the axils of the petiolate broad leaves. In this paper the distal portion of the stem will be termed the 'upper main raceme'; occasionally there is a smaller subraceme immediately below it. Each side-branch has a distal main raceme, and may carry several younger and smaller subracemes. All the racemes are in fact peduncles arising from the axils of sessile leaves or bracts, as distinct from complete side-branches which arise in the broad leaf axils.

In the early stages of development each raceme is a corymbose structure of flower buds, with the older and larger buds on the outside and the young buds and bud

primordia in the centre. As the plant develops the internodes lengthen between the petiolate leaves, and the older raceme axes at the top of the plant extend, carrying the corymbs of flower buds away from the stem. Each separate flower bud is borne on a short pedicel attached to the raceme axis (peduncle). There is a fairly long-lasting green-bud stage, during which the buds swell, and a few days before the first flowers open the sepals of the older buds gradually turn yellow. About five flowers are open at once on any single raceme, and each flower remains open for about 3 days. Flowering proceeds from the base to the tip of the raceme, which gradually lengthens until flowering is complete, and all the petals have fallen. A high proportion of the primordial flower buds at the tip of each raceme never set fruit, either through physiological or genetic limitations or through blossom beetle injury (Kaufmann, 1942).

Flower buds are damaged by both the adult and larval blossom beetles. Most of the damage by adults occurs during May while the buds are still small, and the injured buds wither and fall from the plant leaving only 'blind-stalks' (pedicels). Larval damage is most severe on the distal portion of the racemes during June; this results in withered ovaries and some blind stalks and often gives rise to distinct zones of damage when the racemes are examined after petal fall. Severe larval damage to the tip-buds and raceme rachis occasionally causes a 'tip-drop', which results in a 'pencil-tip' effect instead of a cluster of withered pedicels on the tip of the raceme. Tip-drop was not seen in 1958, but was especially severe on the main racemes in the dry season of 1959. This distinction between adult and larval damage probably depends on the season, the species of plant, and the stage of development of the crop at the time of attack. The zoning effect seems to be clearer on mustard than on other brassica plants, which may explain the conflicting opinions of some of the earlier continental workers on the importance of larval damage.

Sampling methods

A. *Sampling for blossom beetle damage.* Blossom beetle damage may be estimated by counts of blind stalks, but if the larvae have caused severe tip-drop some of these blind stalks will have been lost, and counts of set pods give more reliable figures for comparing treatments. Previous work suggested that the time each raceme came into flower affected the severity of the damage. The sampling methods were evolved as the work progressed; in 1958 the upper and middle main racemes were sampled, but, as this method seemed to overestimate the damage, in 1959 an upper, middle and lower main raceme was sampled from each plant (Text-fig. 1 E, I, II, III). (Hereafter these methods are termed UM and UML respectively.) In 1960 an evaluation of this technique was made on samples of ten plants per plot on a small replicated trial at Trumpington, Cambridge. Counts were made of all the pods and blind stalks on each plant, from which UML main racemes were then subsampled. The results are shown in Table 1. The mean numbers of pods and blind stalks per raceme are included to show that the UML method selects the larger racemes. The UML method covered about one-seventh to one-fifth of the total racemes produced by each plant.

Both methods gave comparable results and though the standard error for whole-plant counts was half that of the UML method, the latter could be performed about six times as quickly. Also the upper, middle and lower raceme subsamples gave three

separate estimates of damage for racemes of different ages, and this was sometimes useful when comparing the damage by blossom beetles on plots treated in different ways.

Ten whole plants sampled from a plot gave reliable results. To achieve similar accuracy it seemed to be necessary to sample racemes from at least twenty plants per plot, and this was the standard sample in 1958. In 1959 raceme-subsamples were taken from twenty-five plants per plot on the Holland trials, and from fifty plants per plot in the Isle of Ely, but there was little advantage in subsampling racemes from fifty rather than twenty-five plants.

Table 1. *Counts of pods and blind stalks/plant compared with UML raceme subsampling for the assessment of blossom beetle damage*

Each figure is the mean of five replicates; ten plants examined from each plot by each method.

	Blind stalks (angles) (%)		Mean no. of pods and blind stalks/raceme		
Estimate	Mean UML	Whole plants	UML method (mean of 3 racemes/ plant)	Whole plants	Mean no. of racemes/ plant
1	31·70	32·62	31·43	17·64	21·7
2	37·67	35·51	30·59	17·51	20·8
3	40·20	37·55	29·23	15·22	15·3
4	39·18	37·36	30·24	15·39	15·4
S.E. of the estimate means	±1·711	±0·818	—	—	—
D.F. (residual)	12	12	—	—	—

B. *Sampling for seed-weevil damage.* Adult seed weevils feed on the flower buds and bracts at the top of the plants, but are not numerous enough to cause significant damage. The eggs are laid singly in the young pods, and normally there is only a single larva in each infested mustard pod; occasionally two, and rarely three, larvae were found. Each larva destroys several seeds and may damage several others during development (see Table 8 below). From random pod samples it is therefore simple to calculate the percentage seed loss (Carlson, Lange & Sciaroni, 1951). In 1958 two separate 100-pod samples were taken from the above-described bulked upper and middle racemes from each plot. The pairs of samples gave very consistent results even at low infestations, and 100 pods was adopted as the standard sample for laboratory examination. In 1959 three separate samples of 100 pods were taken from upper, middle and lower racemes, to discover how the age of the raceme affected the attack; frequently more than one set of samples was examined from each plot. It became evident during July and August, 1959 that the lower racemes were always less heavily infested than the upper racemes from the same plant, and that the younger subracemes were less heavily infested than the main racemes (Table 2). Most of the seed weevil figures given in this paper are overestimates of the true attack but are comparable with one another for assessing the effects of insecticidal sprays.

The extent to which the UML samples overestimate the true attack probably depends on the degree of infestation. On site 1 in 1959 the mean UML pod attack in

July was 25·8%. At harvest 21·2% of these larvae emerged leaving exit-holes (Plate fig. 3). Counts of exit-holes were made on five whole plants from each plot at harvest and the mean count was 15·8%. On this site therefore the UML counts overestimated the true attack by about 5·4%; and the infestation of only the upper main racemes was about 2·5 times that recorded on whole plants.

Table 2. *Seed weevil, percentage pod attack on the control plots of the Isle of Ely trials* 1959

Two control plots on each site or block.
Means of two separate 100-pod samples from U, M and L racemes on each plot.

	Pods infested July (%)					
	Site 1					
	Block 1		Block 2		Site 2	
Plots ...	1	2	1	2	1	2
Racemes						
Upper	49·5	55·5	68·0	69·5	39·0	35·5
Middle	23·5	32·5	43·5	41·5	14·5	35·0
Lower	16·0	21·5	29·5	35·5	11·5	21·0
UML mean	29·67	36·50	43·67	48·83	21·67	30·50

C. *Sampling for the adult insects.* Müller (1941) describes several methods for estimating blossom beetle populations on rape plants, using a sweep-net and 'sucking-tube'. During 1958 and 1959 Müller's net-capture method was used in a slightly modified form on the Holland sites, and a simple and speedy method was devised for use in the Isle of Ely. Single plants were taken at random and shaken into an open tin tray measuring 12 in. × 11 in. × 2 in. deep. The beetles and weevils which remained in the flowers and buds were dislodged by gentle manipulation, and the insects in the tin were tapped into a line down one side and counted quickly. The population of insects was recorded on twenty plants per plot on each occasion, and in early June 1958 standard errors per plant were calculated for several sets of data. When expressed as percentages of their means, the standard errors per plant varied from 7·55% for a population of 12·80 beetles per plant, to 42·00% for a population of 0·75 beetles per plant. Normally, however, there were such large differences between treated and untreated plots that statistical checks were thought to be unnecessary. Furthermore, there were probably large unmeasureable errors due to differences in weather conditions during sampling. On cool days the insects hide themselves in the flower buds and upper leaf axils, and are very difficult to dislodge (see also Müller). In cold wet weather many beetles and weevils take cover in the surface layers of the soil and in the lower leaf axils. Conversely, on hot, bright and calm days a large proportion of the population is airborne, and is not recovered by direct sampling from the plants. The insects are also very sensitive, and may fall to the ground, even before plants are touched (Heymons, 1922). The insect population counts were made only during favourable conditions, and not on rainy days or when the crops were wet.

The tin-method is quick and simple, the net-capture method more laborious, but

the insect population counts obtained by both these methods can be related to a known plant population. For this reason net-sweeping was not used extensively during this work, though on several occasions the tin-method and net-sweeping were compared. It was found that twenty sweeps in the plant heads caught five to six times as many blossom beetles, and four times as many seed weevils, as the tin-method carried out on twenty plants.

When the plants became large and entangled it was difficult to sample separate plants without knocking off the insects, but the critical observations on population build-up and the effects of insecticides were usually completed before the plants became too large to sample.

Field trials on Trowse mustard in 1958 *and* 1959

Spraying trials were carried out on six farms in 1958 and four farms in 1959 in the Isle of Ely and the Holland division of Lincolnshire (Table 3).

In 1958 each field was divided into five plots, to which the treatments shown in Table 4 were allotted at random. All the insecticides were miscible liquid concentrates. On the Holland sites the sprays were applied with a special high clearance, 200 gallon capacity, self-propelled machine with a boom-width of approximately 38 ft. In the Isle of Ely, commercial tractor-drawn 200-gallon capacity low-clearance machines were used. The boom-width varied between 35 and 42 ft., and on site 1 special large diameter wheels with narrow treads were fitted to both tractor and machine to obtain higher clearance and minimize damage to the crop. On all the sites the spray-boom was adjusted to give about 1 ft. clearance above the crop, and the sprays were applied at high volume (90–100 gallons water/acre).

Table 3. *The trial sites in* 1958 *and* 1959

Site reference no.	Location		1958 Date sown	1958 Date harvested	1958 Area harvested/plot (acres)	1959 Date sown	1959 Date harvested	1959 Area harvested/plot (acres)
1*	Linford	Isle of Ely	24 Mar.	17 Sept.	0·27	25 Mar.	21 Aug.	0·57–0·7
2	Manea	Isle of Ely	17 Mar.	8 Sept.	1·33	20 Mar.	25 Aug.	0·17
3	Welney	Isle of Ely	26 Mar.	2 Sept.	0·47	—	—	—
4	Postland	Lincs (Holland)	28 Mar.	16 Sept.	0·26	1 Apr.	27 Aug.	0·53
5	Holbeach	Lincs (Holland)	10 Mar.	13 Sept.	0·25	15 Mar.	24 Aug.	0·19
6	Weston	Lincs (Holland)	15 April	13 Sept.	0·52	—	—	—

*=Two replicates of the experiment on the same field in 1959.

In 1959 dieldrin alone, at the same rate as in 1958, was applied to coincide with certain stages of the crop, which were termed A, B and C. Stage A occurred during mid-May when the upper racemes (Text-fig. 1) were in 'green-bud'; stage B was during late May or early June at 'yellow-bud'; and stage C was in the first or second week of June at 'first open-flower'. On each site or block in 1959 there were seven plots, sprayed at the following stages: A, B, C, AB, BC, and two untreated controls. Each plant-stage lasts several days, and the sprays did not always coincide with the

start of each stage; the dates of application also varied a little from site to site, but are shown for two of the 1959 sites in Text-fig. 3. In 1958 all the sites were sprayed during the yellow-bud stage or shortly after flowering began.

Previous observations on small plots showed that as the insects are very mobile, the population on the whole trial area seemed to be affected by treatment of any one part. It was therefore necessary to use large plots with large discards, but although the plots were 70 ft. wide and at least 2 acres in area in 1958, the insecticidal treatments often had an effect for several yards into the control plots. In 1959 therefore, all the plots were made at least 120 ft. wide and at least 1 acre in area.

The special high clearance machine, used on the Holland sites, caused very little damage to the crops. In 1958 and 1959 some of the plots on sites 2 and 3 in the Isle of Ely were slightly damaged by the tractor and sprayer draw-gear. During harvest, such damaged sections of the plots were avoided, and two 8 ft. combine-swathes from each plot were harvested direct from the standing crops. On site 1 in 1959 two complete combine-swathes round each plot were discarded, and the remainder of the plot was harvested.

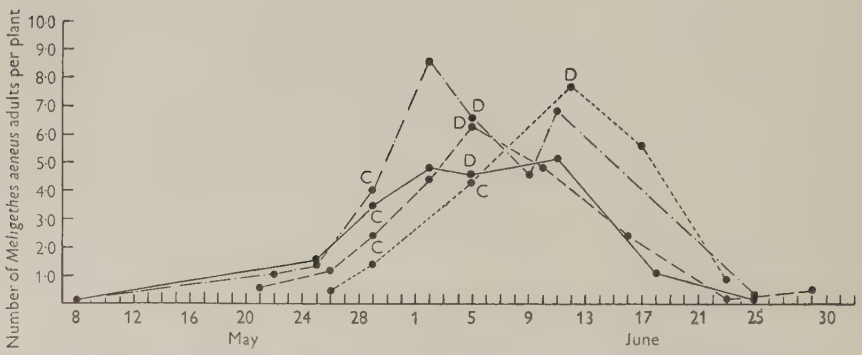
RESULTS

Effects of sprays on the adult insects

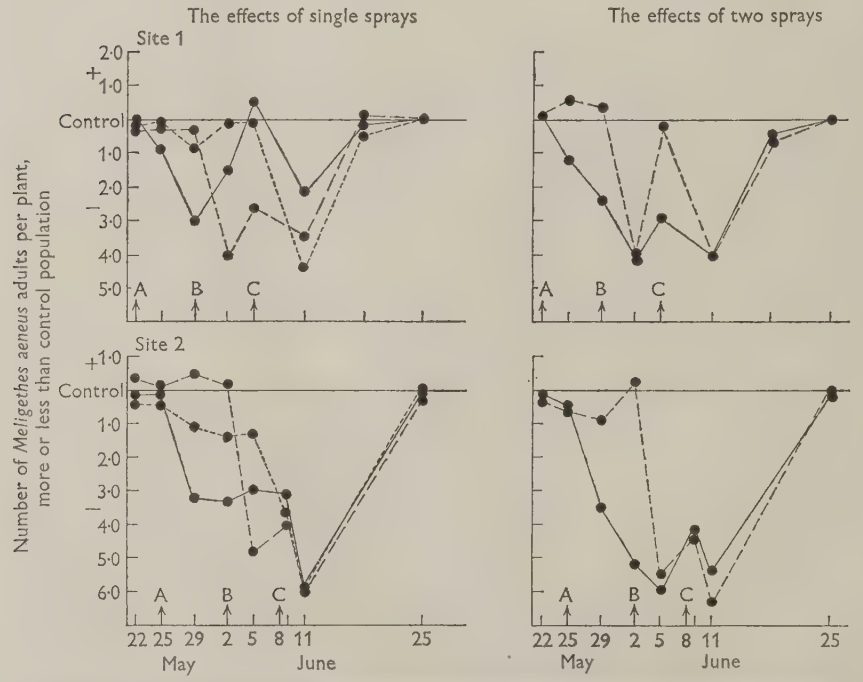
The spraying trials covered a large area and there were often considerable population differences from one side of the field to the other. Risbec (1952*b*) states that seed weevils first invade the borders of the crop, and that constant movement occurs both into and away from the crop during the early period of invasion. Blossom beetles behave in a similar fashion, but seem to spread evenly and more quickly over the crop during May. Frequently the population of both insects on the outer plots remained higher than on plots elsewhere in the field, and on some of the sites there was a distinct population gradient across the field. This was especially marked on site 1 in 1959 which was a very long narrow field. Typical population curves for both insects recorded in 1959 are shown in Text-figs. 2 and 3. There were some variations from site to site but the text-figures show the general trends.

A. *Meligethes aeneus*. The adults invade the crop over a period of some weeks beginning in early May. A peak is reached in late May or early June but in 1958 the counts were not started early enough to record the May build-up. The treatments all had a similar effect in 1958, and the population of blossom beetles was usually lower after spraying than before. However, on some sites the sprays were applied during mid-June when the number of spring adults was declining naturally. Populations fell after spraying on some of the control plots, probably due to the effect of neighbouring treated plots. Although three of the sites were sprayed twice, there was no evidence that two sprays were more effective than only one, probably because in 1958 the sprays were applied too late (Winfield & Gough, 1959).

In 1959 the population rose to a peak and thereafter declined, until at petal fall there were few beetles left (Text-fig. 2) (see also Scherney, 1953). Spraying always had some effect on the population, and usually there were fewer beetles on a plot after spraying than before (Text-fig. 3). The exceptions are difficult to explain other than by local differences in population across the field, although occasionally several days



Text-fig. 2. *Meligethes aeneus* populations on the control plots, 1959 field trials. Forty plants sampled on each occasion (eighty on site 1). Plant stage letters from Text-fig. 1. Site 1 ●—●, site 2 ●- - -●, site 4 ●---●, site 5 ●—·—●.



Text-fig. 3. The effects of spray-treatment on the *M. aeneus* population on two of the 1959 field trials. On spraying days counts were made before spraying (sprays shown by arrows from the date-line), and the curves show the population plus or minus the control. Left, plots treated only once; stage A ●—●, stage B ●- -●, stage C ●----●. Right, plots sprayed twice; stages A and B ●—●, stages B and C ●- -●.

elapsed between spraying and sampling, which gave misleading results during May when the insects were invading the crop. Two sprays were usually more effective than one spray, but none of the treatments in 1959 was consistently successful. Plots sprayed at stages A or B, for example, were often rapidly reinfested as the population was rising, and stage C sprays were too late to prevent early arrivals from becoming established and causing damage.

B. *Ceuthorhynchus assimilis*. Seed weevils were always less numerous than blossom beetles: at maximum populations of each insect the ratio was usually 8–12 blossom beetles to one seed weevil. In 1958, DDT was ineffective against seed weevil (see also Potter & Perkins, 1946; Holz, 1948; and Ankersmit, 1956), but the other three insecticides consistently reduced the weevil population.

In 1959 the peak population was recorded when the first pods were setting or very shortly before (similar to the curves for blossom beetles shown in Text-fig. 2). All the sprays had some effect in reducing weevil numbers and two sprays maintained the population at a lower level than one spray, and again the population curves for seed weevil were similar to those shown in Text-fig. 3 for blossom beetles, though the numbers recorded per plant were much lower. The later sprays reduced the population from the time that the eggs were first being laid in the young pods, after which there is normally no re-invasion (Ankersmit, 1956).

Effect of the sprays on Meligethes aeneus *larvae*

In both years laboratory counts of blossom beetle larvae were made on raceme samples from one of the Isle of Ely sites. In 1958, twenty main racemes were taken from each of the five plots on site 2 on 24 June; and in 1959 twenty-five upper main racemes were taken from each of the fourteen plots on site 1 on 18 June. The results are summarized in Table 4.

Table 4. Meligethes aeneus *larvae on two spraying trials*

1958, Twenty main racemes examined from each treatment.
1959, Twenty-five upper main racemes examined from each treatment.

Sampling date and site	Treatment and % active ingredient	*M. aeneus* larvae, mean/raceme: 1st instar	2nd instar	1st and 2nd instar	
24 June 1958 Site 2	0·019 % Parathion	2·25	1·40	3·65	Plots sprayed 6 and 15 days before sampling
	0·025 % γ-BHC	0·90	0·65	1·55	
	0·038 % Dieldrin	2·45	0	2·45	
	0·094 % DDT	3·60	0·90	4·50	
	Control	5·20	9·00	14·20	No treatment
					Plots sprayed:
18 June 1959 Site 1	Stage A*	6·44	9·22	15·66	27
	Stage B*	2·20	1·84	4·04	20
	Stage C*	1·76	2·72	4·48	13
	Stages AB*	1·44	1·54	2·98	20 and 27
	Stages BC*	3·08	0·10	3·18	13 and 20
	Control	4·05	6·81	10·86	No treatment

Days before sampling

* All the sprays in 1959 were 0·038 % dieldrin.

The sprays reduced the numbers of larvae, and those recorded on the treated plots probably came from eggs laid after spraying. In 1959 the double sprays were more effective than the single sprays, and the larvae were less numerous on more recently sprayed plots than on plots sprayed some time before. Frequently the effects of blossom beetle larval damage were visible in the field, especially where a control plot and a late sprayed plot were side by side. During mid-June the control plots and those treated once at the green-bud stage were distinctly greener than the later sprayed plots. There were more withered raceme tips and less open flowers on the former and the latter were clearly more yellow in colour.

Comparative pest damage and yields of seed

For the purposes of comparing damage by blossom beetles the counts of blind stalks are expressed as percentages of the total pods and blind stalks; the figures are average values for UM and UML racemes in 1958 and 1959, respectively. The results of the raceme and pod examinations which were carried out in the laboratory during June, July and August are summarized in Table 5. The figures obtained are comparative only, and are probably overestimates of the true losses caused by each pest.

Table 5. *Pest damage and yields of seed on the spraying trials in* 1958 *and* 1959

Damage estimates are means for UM and UML racemes in 1958 and 1959 respectively.

		Parathion	Dieldrin	γ-BHC	DDT	Control	S.E. of treatment means (residual D.F. 20)
1958							
M. aeneus damage blind stalks (%)	Percentage	43·0	40·5	38·0	39·0	55·6	—
	Angle	40·99	39·53	39·06	38·62	48·21	±1·661
C. assimilis pods infested at harvest (%)*	All pods	18·5	15·5	18·2	23·8	26·7	—
	Pods with exit-holes	17·3	9·0	12·3	16·2	19·8	—
Yield of seed, cwt./acre at 85 % dry matter	—	10·75	10·93	10·12	9·90	9·06	±0·112

		A	B	C	AB	BC	Control	Control	S.E. of treatment means (residual D.F. 30)
Stage of spraying	...								
1959									
M. aeneus damage blind stalks (%)	Percentage	38·7	41·2	36·2	29·0	33·1	43·0	50·7	—
	Angle	38·44	39·94	36·97	32·56	35·13	40·98	45·39	±2·789
C. assimilis infested pods July (%)	Percentage	24·5	18·8	10·8	17·5	10·2	28·3	50·8	—
	Angle	29·65	25·69	19·18	24·69	18·66	32·15	45·45	±1·249
Yields of seed, cwt./acre at 85 % dry matter	—	18·39	18·77	19·39	18·20	18·44	18·12	17·47	±0·870

* Means for three sites.

In 1958 all the sprays increased the yields of seed. Blossom beetle damage on the six sites ranged between 46·8 and 39·3 %, and yields of seed between 11·89 and 8·74 cwt. per acre. It seems that adult blossom beetle damage was only partly controlled,

and that most of the differences in 1958 were probably due to a control of the larvae of blossom beetles and of the adult seed weevils before they laid their eggs. Dieldrin seemed to be the most promising of the insecticides tested and was therefore selected for use in 1959.

The yields were very much higher in the warm and dry season of 1959, than they were in 1958 which was cool and wet. There were also much larger differences between sites both in incidence of the pests and in yield. Blossom beetle damage ranged between sites from 48·1 to 30·2%, and the pod attack by seed weevil larvae from 28·9 to 12·6%. The highest mean yield per site was 20·43 cwt., and the lowest 16·07 cwt. per acre. There were no significant yield differences between the treatments, probably because of the large variations between sites, though the sprayed plots were consistently better than the controls. Two sprays reduced blossom beetle damage more than one, probably because both adults and larvae were controlled. A single spray at any date did not give an adequate control of both adult and larval blossom beetle damage, though of the three single sprays, that applied at stage C seemed to be the best. This spray also consistently gave a satisfactory control of the attack by seed weevil larvae.

The 1959 *survey*

The main results of the survey of twenty-six Trowse mustard crops are summarized in Table 6. The fields were all visited during June to record data on cropping and spraying, and again in July and August to collect samples of plants. There were very few crops in each treatment group so the average figures must be viewed with caution, and moreover it could be argued that the general level of husbandry of farmers who did not spray might be lower than that of farmers who did spray.

Table 6. *Summary of pest damage and yields*, 1959 *survey*

Pest damage obtained from UML raceme subsamples.
Percentages rounded to the nearest whole number.

Treatment	No. of crops	Acreage	*M. aeneus* blind stalks (%)	*C. assimilis* infested pods (%)	Yield in cwt./acre at 85% dry matter
No treatment	4	33	58	51	9·5
Parathion × 1	2	30	31	19	13·5
DDT × 1	4	58	37	28	12·3
DDT × 2	2	13	29	40	17·0
Dieldrin × 1	10	130	29	22	16·0*
Dieldrin × 2	1	10	16	59	23·0
Dieldrin × 3	2	24	15	21	23·0
Various × 3	1	10	19	2	21·3

* Average yield of eight crops.

The highest pest attacks and the lowest yields were associated with no treatment; intermediate blossom beetle damage and yields were associated with DDT or single treatments of other insecticides; and the lowest blossom beetle attacks and the highest yields with two or more dieldrin treatments or three mixed treatments. The high seed weevil attack on the crop sprayed twice with dieldrin is explained by the fact that spraying ceased on 23 May, before egg-laying began (Table 5 above).

Separate counts of blind stalks and withered ovaries due to adult and larval blossom beetles were made on four of the fields in the survey. One of the crops was unsprayed, one was sprayed once early, a third sprayed once late, and the fourth crop was sprayed twice, both early and late. The results are shown in Table 7.

Table 7. *Pest damage and yield of four crops of Trowse mustard* 1959 *where the damage by* Meligethes aeneus *adults and larvae was recorded separately*

Date of sowing	Treatment	*M. aeneus* damage (% blind stalks) average of UML raceme samples		*C. assimilis* pods infested (%)	Yield of seed (cwt./acre)
		Adult damage	Larval damage		
7 Mar.	No treatment	53·46	Very large*	65	9·1
7 Apr.	Dieldrin × 1. 21 May	12·87	21·13	18	14·6
3 Apr.	Dieldrin × 1. 6 June	32·00	13·50	10	15·8
21 Mar.	DDT × 2. 1 May and 1 June	17·90	8·70	47	17·5

* Tip-drop very severe, larval damage could not be estimated.

The unsprayed crop suffered very severe blossom beetle damage. The early spray gave a better control of adult damage than the later spray, which gave a better control of larval damage; and the crop which received two sprays suffered the least damage of the four. The yields of seed seemed to follow fairly closely the figures for pest damage.

DISCUSSION

It is clear that with so many dynamic features, such as weather, crop growth and the activity of the pests all interacting, to elucidate the importance of each pest would take many years. It is unlikely that there would be a simple relationship between yield and the number of adult pests at any given time. It has, however, been possible to make some progress in sorting out the effect of seed weevil larvae, and in relating pod damage to yield.

The seed weevil figures obtained from UML samples were overestimates because pod-samples were taken from the older and more heavily infested racemes (Table 2 above). It is unlikely, however, that the plants compensate for seeds destroyed by weevil larvae. Table 8 shows the numbers of seeds per pod, the number of seeds damaged and destroyed per larva, and the percentage seed loss in the infested pods, at several different sites in 1958 and 1959.

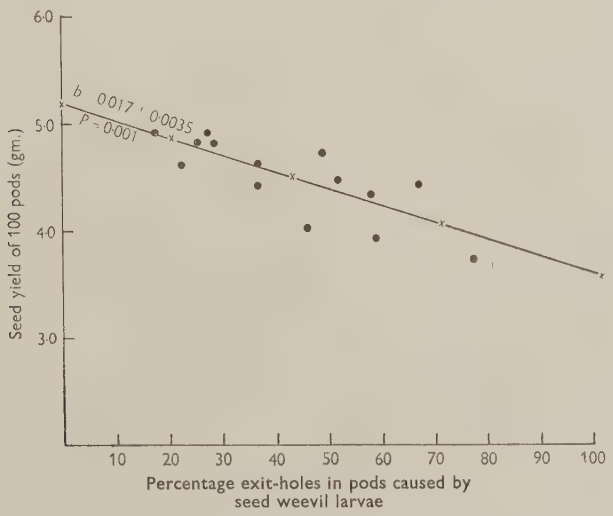
The average number of seeds per pod on Trowse mustard seems to remain fairly constant at about 14·5. The percentage seed loss in the infested pods varied both with site and season, and it is suggested that this variation is related to seed size. In 1959, for example, the average yields of seed were higher than in 1958: the individual seeds were probably larger in 1959 and each weevil larva consumed 0·76 less seeds than in 1958.

Samples of 100 pods were taken at harvest from twenty-five upper racemes from each plot of site 1, where there were fourteen plots. The pods with exit-holes (larvae

Table 8. *Number of seeds destroyed in each pod by seed weevil larvae at various sites, not including seeds damaged or destroyed by larvae which were parasitized, or died prematurely and did not emerge from the pods*

Site	No. of infested pods examined	Damaged and destroyed seeds	Total seeds	Seed loss in infested pods (%)
Spraying trial 1, 1958	185	854	2,704	31·58
2, 1958	91	370	1,530	24·18
3, 1958	152	717	2,156	33·26
Trumpington plots 1958	45	193	627	30·78
Benwick, Isle of Ely plots 1958	117	572	1,571	36·41
Total	590	2,706	8,588	—
Mean per pod	—	4·49*	14·56	31·51
Spraying trial 1, 1959	564	2,133	8,169	26·11
2, 1959	151	655	2,330	27·25
Total	715	2,788	10,499	—
Mean per pod	—	3·73*	14·54	26·55

* Numbers of seeds damaged and destroyed per larva.



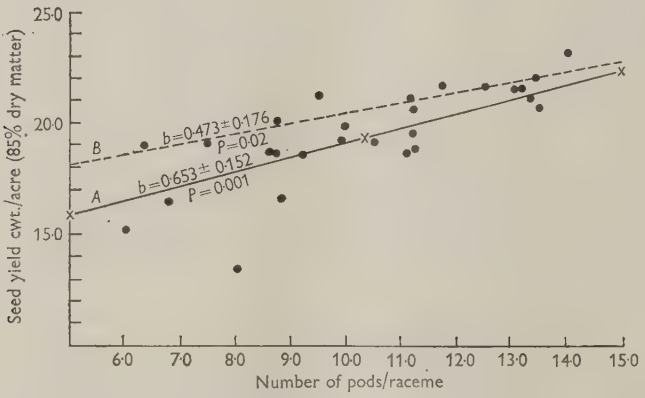
Text-fig. 4. Regression of yield/100 pods on percentage exit-holes caused by seed weevil larvae, site 1, 1959.

emerged) were opened, and the seed counted, after which the seed from the whole sample of 100 pods was weighed. Text-fig. 4 shows the regression of yield in grams on the number of exit-holes per 100 pods.

For 100 exit-holes in 100 pods it is calculated that the yield would have been reduced by 31·77%. At site 1, 26·11% of the seeds were damaged or destroyed in pods

with exit-holes (Table 8). The discrepancy of 5·66 % between the two estimates for seed loss may be due to non-emerged larvae. Such larvae would contribute to the reduction in yield (Text-fig. 4), but were not recorded in direct seed counts obtained only from pods with exit-holes (Table 8). Doucette (1948) discusses premature larval mortality due to parasitism and other causes.

The plants may partly compensate for flower buds lost through injury by *M. aeneus*, by producing new racemes, extra buds on existing racemes or by increasing the size of seeds in the remaining pods. It does not necessarily follow, therefore, that because a figure of 40 % blind stalks was recorded, that 40 % of the yield was lost. A range of estimates for blossom beetle damage on the various treatments was therefore obtained from counts of set pods. Immediately before harvest 1959, samples of five plants were



Text-fig. 5. Regression of yield on pods/raceme, four replicates of the 1959 field trials. 'Pods/raceme' obtained from counts of pods and racemes on five plants/plot. Line *A* not corrected for seed losses due to *C. assimilis* larvae; line *B* corrected for seed losses due to *C. assimilis* larvae. The points on the figure refer only to line *A*.

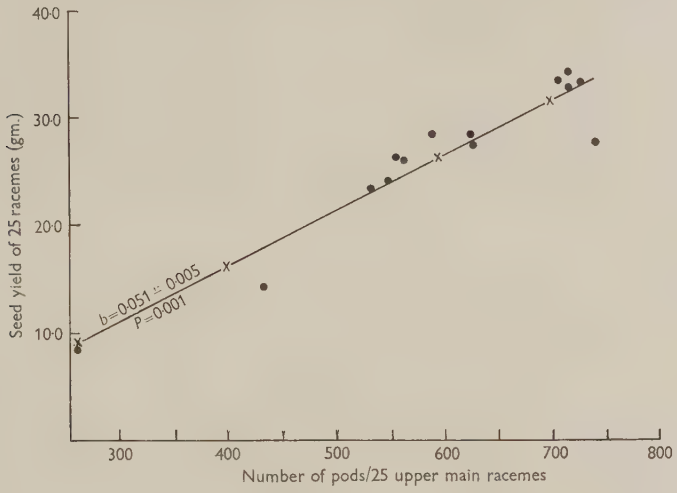
taken at random from each plot on three of the spraying trial sites. The mean number of pods per raceme was calculated for each plot from counts of racemes and set pods, and the regression of yield in cwt. per acre on the number of pods per raceme is shown as a solid line in Text-fig. 5. The yields can be corrected for seed weevil losses as shown by the broken line in Text-fig. 5. (The individual yields were corrected by 2·9 per 10 % infested pods in July, which overestimated the true attack, and therefore the corrected line should be somewhat lower than shown in Text-fig. 5.)

The lower yields were associated with high percentages of blind stalks and low numbers of pods per raceme; the corrected line in Text-fig. 5 therefore shows the relationship between yield and pods per raceme remaining after blossom beetle damage.

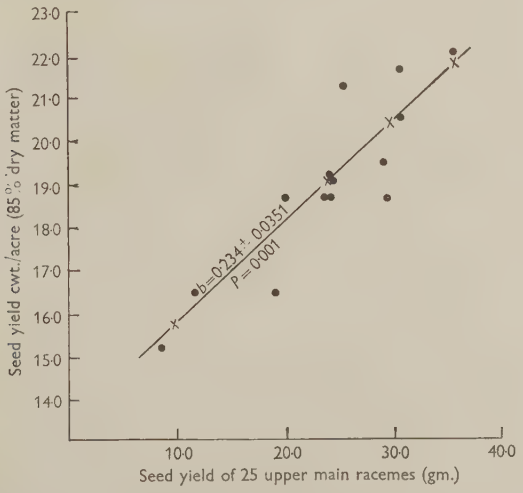
It was hoped that upper main racemes could be used as 'indicator' samples to assess the effect on yield of both pests. Although regressions were calculated for yield on pods per upper raceme, it was obvious that both the yields and the numbers of pods varied with season, and that many years' observations would be necessary to show any

worthwhile correlation. However, a regression of yield on pods per twenty-five upper main racemes is shown in Text-fig. 6 for site 1. The individual plot yields have been corrected for seed weevil losses at the rate of 3·177 % loss per 10 % infested pods (from Text-fig. 4).

The effect of blossom beetles is exaggerated on the upper racemes but Text-fig. 6



Text-fig. 6. Regression of yield on pods/twenty-five upper main racemes, site 1, 1959. Corrected for seed losses due to *C. assimilis* larvae at 3·177/10 % exit-holes in pods.



Text-fig. 7. Regression of yield/plot in cwt./acre on yield of twenty-five upper main racemes (g.), site 1, 1959.

shows a good correlation. The plots most severely damaged by blossom beetles gave the poorest yields: for every increment of 100 pods the yield increased by 5·07 g.

Text-fig. 7 shows a less significant, but positive correlation between the yield of twenty-five upper racemes in grams and the yield in cwt. per acre for the fourteen plots at site 1.

If there had been more replication it is possible that an even better correlation would have been achieved, but it is evident that further work is necessary on the use of upper main racemes as 'indicator' samples.

More than one spray is necessary to control the damage by *M. aeneus*. The best control of the attack by seed weevil larvae in the pods was achieved in the trials by relatively late sprays, when there were open blossoms in the crop. Although Trowse mustard is a self-fertile variety more evidence is needed both of the economic value of the later sprays, and of their effects on bees and the parasites and predators of the pests before spraying during the blossom period could be safely recommended.

I am very grateful to Dr H. C. Gough for his constant advice, and his help in the preparation of this paper, and to Miss E. C. Mason who carried out the trials in Lincolnshire (Holland). Thanks are also due to Mr P. W. Dawson who conducted the survey in 1959, financed by Messrs J. and J. Colman Ltd., of Norwich; to J. W. Chafer Ltd., of Doncaster who supplied the insecticides for the Isle of Ely trials; and to the many farmers for their helpful co-operation. Grateful acknowledgement is made to Dr C. Potter and his staff, of Rothamsted Experimental Station, and my colleagues in the N.A.A.S., especially the County Staffs of Norfolk and the Isle of Ely, for their helpful suggestions and assistance. Finally I would like to thank Miss J. A. Hewitt, Miss P. M. Yates, Mr T. R. Williams and Mr A. G. Sherif for their help in examining the numerous plant samples, and Mr W. E. Dant for taking the photographs of damage.

REFERENCES

ANKERSMIT, G. W. (1956). The biology and control of the colza seed-pod weevil (*Ceuthorhynchus assimilis* Payk) and the brassica pod midge (*Dasyneura brassicae* Winn). *Inst. v. Plantenziekt. Onderz. Wageningen. Med.* **132**, 59 pp.

BAUERS, C. (1954). Versuche mit Toxaphen-Staub zur Feststellung der Bienengefährlichkeit und der Wirkung gegen Kohlschotenrüssler und Rapsglanzkäfer. *Anz. Schädlingsk.* **27**, 35.

BONNEMAISON, L. (1951). Protection des Crucifères oléagineuses contre les Méligèthes. *C.R. Acad. Agric. Fr.* **37**, 441.

BONNEMAISON, L. (1952). Méthodes de lutte à appliquier contre le charançon des siliques (*Ceuthorhynchus assimilis* Payk). *C.R. Acad. Agric. Fr.* **38**, 759.

BOURON, H. (1955). Observations sur une opération de lutte contre le Ceuthorrhynque du Colza en 1954. *Phytomya*, **66**, 16.

CARLSON, E. C., LANGE, W. H. JNR. & SCIARONI, R. M. (1951). Distribution and control of the cabbage seedpod weevil in California. *J. econ. Ent.* **44**, 958.

DOUCETTE, C. F. (1948). Field parasitization and larval mortality of cabbage seed-pod weevil. *J. econ. Ent.* **41**, 763.

FREY, W. (1950). Über die Beziehungen zwischen der Wirksamkeit chemisches Bekämpfungsmittel und dem Entwicklungszustand des Rapsglanzkäfers (*Meligethes aeneus* F.). *Z. angew. Ent.* **31**, 609.

GODAN, D. (1952). Untersuchungen zur Bekämpfung der Kohlschotenrüssler und Kohlschotenmücken-Larven mit Ester und Gamma-Hexa-Mitteln. *Anz. Schädlingsk.* **25**, 33.

Fig. 1

Fig. 2

Fig. 3

(*Facing p.* 555)

Hascoët, M. (1953). Étude sur les traitements aériens par poudrage. II. Traitments contre le méligèthe du colza. *Ann. Épiphyt.* **4**, 307.

Herrström, G. (1951). Sambandet mellan blygrå rapsvivelns ålder och känslighet för hexaklorhaltiga preparat. *Växtskyddsnotiser, Stockh.* 1951 (2–3), p. 17. (*Rev. appl. Ent.* (A), **40**, 216, 1952.)

Heymons, R. (1922). Mitteilungen über den Rapsrüssler *Ceuthorhynchus assimilis* Payk und seinen Parasiten *Trichomalus fasciatus*. *Z. angew. Ent.* **8**, 93.

Holz, W. (1948). Freilandversuch mit neuen Kontaktinsektiziden gegen Rapsglanzkäfer und Kohlschotenrüssler unter gleichzeitiger Beobachtung des Befalls durch die Kohlgallmücke. *Anz. Schädlingsk.* **21**, 23. (*Rev. appl. Ent.* (A), **38**, 346, 1950.)

Jary, S. G. & Carpenter, K. (1948). Insect pests of brassica seed crops in Romney Marsh, Kent. *Ann. appl. Biol.* **35**, 134.

Kaufmann, O. (1942). Ueber Reaktionen der schossenden Rapspflanze auf Rapsglanzkäferfrass und andere Schäden. *Z. PflKrankh.* **52**, 486.

Müller, H. J. (1941). Beiträge zur Biologie des Rapsglanzkäfers *Meligethes aeneus* F. *Z. PflKrankh.* **51**, 385.

Potter, C. & Perkins, J. F. (1946). Control of brassica pests by DDT. *Agriculture*, **53**, 109.

Risbec, J. (1952*a*). Le Ceuthorrhynque des siliques du Colza—Formule pour la determination de la date des traitements insecticides. *C.R. Acad. Agric. Fr.* **38**, 353.

Risbec, J. (1952*b*). De l'Évolution des insectes nuisibles au Colza et des moyens de lutter contre eux. *C.R. Acad. Agric. Fr.* **38**, 403.

Scherney, F. (1953). Zur Biologie der an Raps vorkommenden Meligethesarten. *Z. PflBau*, **4**, 154. (*Rev. appl. Ent.* (A), **44**, 253, 1956.)

Winfield, A. L. & Gough, H. C. (1959). Pests of mustard seed crops. *Agriculture*, **65**, 554.

EXPLANATION OF PLATE

Fig. 1. Blossom beetle damage on Trowse mustard showing separate zones of adult damage (A), and larval damage (B) ($\times \frac{1}{3}$).

Fig. 2. 'Tip-drip' (arrowed), caused by severe blossom beetle larval damage showing 'pencil-tip' effect. Mid-July ($\times \frac{2}{3}$).

Fig. 3. Exit holes in Trowse mustard pods caused by seed weevil larvae ($\times$ 1).

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The list of speakers and titles of papers presented in 1960 are given on pp. 387–8 of vol. **49**. Meetings in 1961 were held on 20 January and 10 February at the Natural History Museum, London, and on 10 March at Rothamsted.

Abridged versions of the following papers are printed below:

Meeting of 11 *March* 1960

The frit fly—a denizen of grassland and a pest of oats. By Dr T. R. E. Southwood and Dr W. F. Jepson

Meeting of 10 *March* 1961

Varietal differences in some physiological characters of wheat. By Mr F. G. H. Lupton

Analysis of ecotypic differences in tall fescue (*Festuca arundinacea* Schreb.). By Mr B. N. Chatterjee

Ann. appl. Biol. (1961), **49**, 556–557

The frit fly—a denizen of grassland and a pest of oats

By T. R. E. Southwood and W. F. Jepson
Imperial College, London, S.W. 7

Whereas with most insect pests the density of the host plant is much greater in the crop than in the wild habitat, with insect pests of cereal whose wild habitat is grassland the opposite condition would appear to exist. It is therefore of general, as well as specific, interest to compare the state of the population of the frit fly, *Oscinella frit* L. (Dipt. Chloropidae), in grassland with that when it is in oats. Its condition in these two habitats is found to differ in two main respects:

(*a*) There are many Dipterous stem borers in grasslands, several as abundant as the frit fly, which is, however, the only species to attack oats.

(*b*) The population level of the frit fly in the grasslands remains more or less steady, being if anything highest in the over-wintering generation; in the oat crop the adults emerging from the panicles are about two hundred times more numerous than those that invade the field in the spring.

It is suggested that there are two, not mutually exclusive, sets of factors which might account for the steady population state in the grasslands and yet are absent or reduced in the oat crop. They are:

(1) The attacks of natural enemies, parasites and predators, and interspecific competition with other Dipterous stem borers.

(2) A relative shortage of suitable sites for development; these may be influenced by climate.

Work is in progress to assess the relative importance of each of these sets of factors. Results to date strongly suggest that the second is the more important. In the grasslands that part of the population living in tillers is limited by the number of tillers in the correct state for colonization and the difficulty of finding these amongst many that are unsuitable. The frit fly is also able to develop in ripening grass grains of a sufficiently large size, there are between 30 and 80 million such grains in an oat field; of the wild grasses probably only *Bromus* is sufficiently heavy-grained to serve as a host and this seldom occurs as a pure stand over a large area.

The frit fly is a highly mobile insect and under certain circumstances at least about three-quarters of the adult population leave the field each day. Large numbers are carried in the upper air current, like aphids. The frit fly population in grasslands and oats must therefore be considered as potentially freely interchangeable over a large area.

This conclusion, if correct, justifies an attempt to estimate the total productivity of adult frit flies of various types of grassland and oat crops in England and Wales.

Virtually all adult flies of the overwintering generation emerge in early spring in grasslands; the resultant tiller generation is also predominantly of grassland origin, although conspicuous populations are produced from oat fields. But in the panicle generation the numbers produced from oat grains are so large as to greatly exceed the population from grasslands and in fact produce a considerable increase in the overall national frit fly population.

Ann. appl. Biol. (1961), **49**, 557–560

Varietal differences in some physiological characters of wheat

By F. G. H. Lupton

Plant Breeding Institute, Trumpington, Cambridge

In seeking to breed improved varieties of any crop it is most desirable that the plant breeder should have as complete a knowledge as possible of the varieties of his crop available for hybridization. This is particularly important when handling a crop such as wheat, which has been subject to improvement by conscious selection or otherwise for many centuries, so that a very wide range of material is available which may be used by the plant breeder. In addition, wheat is a self-pollinating crop the varieties of which are true breeding homozygous lines. Before he can make any improvements the breeder must, therefore, himself create the mixed population within which to make his selection.

Varietal hybridization remains by far the most important method by which such variation is created. The choice of parents for hybridization is often a matter of considerable difficulty, however, especially when breeding for improvement in yielding capacity, or any other character determined by a complex genetic system. In dealing with such characters it is often impossible to select the best parental combinations by consideration of the potential parental varieties *per se*. It is therefore necesssary to make a range of crosses and to select the parental combinations on which to concentrate attention by consideration of the behaviour in early generations of the progenies obtained.

The need for such a procedure is an indication that our knowledge of the parental varieties is incomplete. A better understanding of the physiological characters of these varieties at successive stages in their development, and of the relation of these characters to yielding capacity, would however greatly simplify the problems involved in the choice of the best parental combinations. There is little doubt that the physiological characters determining yield are not strictly additive, but that high expression of certain characters is necessarily correlated with low expression of others. Nevertheless, it should be possible to select the better parental combinations by considering the physiological characteristics of each variety and then choosing parents with complementary features which could subsequently be selected as recombinants in the hybrid population.

The experiments described in this paper were designed to give evidence on differences in photosynthetic activity of six wheat varieties at different stages in their development. The varieties used were the winter wheats Cappelle, Heine VII, Holdfast, Hybrid 46 and Minister and the spring wheat Peko. Both experiments were carried out on autumn-sown plots in the field. It is, however, planned to repeat the experiments under controlled conditions in a growth chamber.

The development of the wheat plant may be divided into two phases, the first lasting from germination to ear emergence and the second from ear emergence until harvest time. Estimates have been made of net assimilation rate throughout the earlier phase. It is, however, extremely

difficult to estimate the photosynthetic area of an ear of wheat, to say nothing of the complicating factors of shading and of the loss in photosynthetic activity during ripening. An indirect approach has therefore been adopted, to determine the contribution of photosynthesis in the ear to grain weight at harvest.

ESTIMATION OF NET ASSIMILATION RATE

Estimates of net assimilation rate (N.A.R.) were obtained from measurements of photosynthetic area and dry weight made in late November, early February and then at successively shorter intervals until ear emergence. Photosynthetic areas measured included leaves, leaf sheaths and stems; dry weight samples comprised whole plants, with as much root as could be raised with a hand fork. The data obtained are given in Table 1 from which it will be seen that there are significant varietal differences in N.A.R. between the varieties investigated, the most prominent features being the high value for the spring wheat Peko, the lower value for Holdfast and the sudden increase in N.A.R. shown by Heine VII in late April and early May, a time when this variety, which is relatively slow growing during the winter months, shows a sudden increase in growth rate.

Table 1. *Net assimilation rates of wheat varieties* (*mg./cm.²/day*)

	30. xi. 59–3. ii. 60	3. ii. 60–22. iii. 60	22. iii. 60–19. iv. 60	19. iv. 60–3. v. 60	3. v. 60–18. v. 60	18. v. 60–26. v. 60
Holdfast	0·087	0·183	0·258	0·327	0·341	0·145
Cappelle	0·080	0·222	0·320	0·277	0·323	0·000
Minister	0·095	0·228	0·250	0·393	0·289	0·117
Hybrid 46	0·096	0·206	0·285	0·374	0·314	0·016
Heine VII	0·109	0·223	0·273	0·460	0·298	0·217
Peko	0·100	0·258	0·356	0·324	0·391	0·237
L.S.D. ($P = 0·05$)	0·017	0·026	0·071	0·095	0·097	0·113

The figures obtained during the period immediately before ear emergence are clearly more erratic than those obtained earlier in the season and must be treated with some scepticism, as the relation between leaf area and plant dry weight is clearly not linear at this time. It is, however, interesting to note the very marked fall in N.A.R. of all varieties shortly before ear emergence amounting to a fall to zero in the case of Cappelle and Minister. This observation suggests that considerable energy is required to bring about the very rapid growth of the ear as it emerges, and that this utilizes most of the products of photosynthesis at that time. No great importance should be attached to the observation that the growth of Cappelle and Minister appears to be halted, while that of the other varieties is merely reduced. This probably reflects the times of the sampling, which apparently coincided exactly with the period of greatest energy requirement of Cappelle and Minister, but not of the other varieties. It is planned to repeat the experiment with observations at much closer intervals in order to obtain further evidence at this critical period.

ESTIMATION OF PHOTOSYNTHESIS IN THE EAR

Most previous estimates of the contribution of photosynthesis in the ear to grain weight have been based on techniques in which the ears have been enclosed in dark containers to prevent photosynthesis, and the grain weights compared with untreated controls (e.g. Asana, Saini & Ray, 1958; Asana & Mani, 1950; Archbold, 1942; Watson & Norman, 1939). The effects of such treatment on respiration and transpiration must be very drastic, however, and so, as an alternative, the technique adopted here is a modification of that evolved by Buttrose & May (1959), in which the lower parts of the plants are shaded, and the ears alone allowed to continue photosynthesis. The number of grains developing is limited to ten on some plants and to thirty on others. When the lower parts of a plant are shaded, the reserves of carbohydrate and other foods in these parts will be in short supply. These limited reserves will be translocated to the developing grains. If ten grains only are allowed to develop, they will therefore each get a bigger share of these reserves than if thirty grains are allowed to develop.

Buttrose & May suggest that these reserves are equally distributed amongst the developing grains. By comparing the grain weights of shaded plants with thirty grains with those of shaded plants with ten grains, it is possible to determine the weight of reserves translocated from the shaded parts of the plants, and hence to obtain an estimate of the contribution to grain weight of photosynthesis in the ear.

When the number of spikelets on a wheat ear is reduced, there is a tendency for the surviving spikelets to set an unusually large number of grains. As these grains are frequently small, they might have led to some distortion of the results obtained. All spikelets of ears with thirty grains as well as those with ten grains were therefore reduced to two flowers each by removal of the central flowers shortly after ear emergence. Rudimentary spikelets at the tip and base of the ear were in all cases removed.

Table 2. *Grain weights of shaded and unshaded plants (mg.)*

	Stems shaded		Stems not shaded		Contribution to grain weight of photosynthesis in the ear
	10 grains/ear	30 grains/ear	10 grains/ear	30 grains/ear	
Holdfast	32·2	20·5	37·9	44·8	15·0
Cappelle	34·4	19·2	56·0	60·3	10·0
Minister	43·2	22·6	54·9	55·5	12·2
Hybrid 46	42·3	20·4	51·9	57·6	10·0
Heine VII	42·2	25·7	50·0	54·1	17·4
Peko	53·1	38·7	55·5	59·4	31·1
				L.S.D. ($P = 0{\cdot}05$)	6·3

The mean grain weights of shaded and control plants with ten and thirty grains per ear, respectively, are given in Table 2. In order to ensure comparability of results in determining grain weight, the lowest six grains on each ear were weighed in each case. The varietal differences in grain weight, and the considerable differences due to treatment, are obvious, and were significant at a very low probability.

These figures provide the evidence necessary to determine the contribution of ear photosynthesis to grain weight. For each variety we have the relation: $W = E + (R/n)$, where W is the grain weight, E is the portion of W due to ear photosynthesis, R is the total reserves translocated from the shaded parts and n is the number of grains.

This gives a pair of simultaneous equations for each variety, from which E, the part of the grain weight due to ear photosynthesis, can be calculated. The figures obtained are given in the right-hand column of Table 2. It will be noted that significant varietal differences in ear photosynthesis were obtained, and that the figure for the variety Peko is much greater than that for the other varieties tested.

In order to obtain estimates of the proportion of final grain weight due to photosynthesis in the ear, it is necessary to obtain estimates of grain weight of the unshaded control varieties. The figures obtained are given in Table 2 and show a very surprising feature. While in the shaded plots the grain weight of the thirty-grain samples is less than that of the corresponding ten-grain samples, in the controls the reverse is the case. The difference, moreover, is highly significant, $P < 0{\cdot}001$. It appears therefore that the 'surgery' required to reduce the spikelet number, or possibly that required to remove the central flowers from each spikelet, has had an adverse effect on the grain, and that this effect is more severe on the ears with ten grains than on those with thirty grains. There is no reason why this effect, which may be associated with a premature ripening of the ears in which only ten grains were allowed to develop, should be confined to the control plants. In all probability a similar adverse effect was induced in the shaded plots. This would have the effect of reducing the difference between the grain sizes of shaded plants with ten and thirty grains, so that false estimates would have been obtained of the ear photosynthesis. In view of these complicating factors, no estimates of the percentage of normal grain weight due to ear photosynthesis have been made. The figures obtained however suggest that this is of the order of 50 % for Peko and of the order 20–30 % for the other varieties tested.

The demonstration of varietal differences in N.A.R. and in photosynthesis in the ear is of very considerable interest to the plant breeder, and experiments are now being initiated to determine the mechanism of inheritance of these characters. We may now look forward to future refinements of the techniques for determining varietal differences in physiological characters and to demonstrations of the relationship of such characters to yielding capacity.

REFERENCES

ARCHBOLD, H. K. (1942). Physiological studies in plant nutrition. XIII. Experiments with barley on defoliation and shading of the ear in relation to sugar metabolism. *Ann. Bot., Lond.*, N.S. **6**, 487.

ASANA, R. D. & MANI, V. S. (1950). Studies on physiological analysis of yield. I. Varietal differences in photosynthesis in the leaf, stem, and ear of wheat. *Physiol. Plant.* **3**, 22.

ASANA, R. D., SAINI, A. D. & RAY, D. (1958). Studies on physiological analysis of yield. III. The rate of grain development in wheat in relation to photosynthetic surface and soil moisture. *Physiol. Plant.* **11**, 655.

BUTTROSE, M. S. & MAY, L. H. (1959). Physiology of ear development in barley. I. The source of carbon for the developing barley kernel. *Aust. J. biol. Sci.* **12**, 40.

WATSON, D. J. & NORMAN, A. G. (1939). Photosynthesis in the ear of barley, and the movement of nitrogen into the ear. *J. agric. Sci.* **29**, 321.

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Analysis of ecotypic differences in tall fescue (*Festuca arundinacea* Schreb.)

BY B. N. CHATTERJEE

Grassland Research Institute, Hurley, Berkshire

In Britain the variations in solar energy and in temperature between summer and winter give a markedly seasonal character to pasture production, with peaks in the summer months and low production in the winter. The winter feeding of stock has to depend largely on conserved fodder, stored roots, concentrates and kale which will stand through the winter months in the field. Pasture agronomists have always sought to reduce the winter shortage of feed by selecting species that can make some new growth in the winter. The Aberystwyth variety of tall fescue S 170 has been considered to be among the more productive grasses in autumn and early spring. Forms of tall fescue from Algeria and Morocco appear superior to S 170 in winter growth and in freedom from 'winter burn'. The work described in this paper was undertaken in order to determine the morphological and physiological basis of this winter production.

CLIMATIC CONDITIONS IN THE REGION OF THE ORIGIN OF THE MEDITERRANEAN FORMS

The three Mediterranean forms studied come from sites 200, 2000, and over 5000 ft. above sea level. The annual rainfall ranges from 30 in. at the lowest to 10 in. at the highest site; and it occurs in the winter months, when the monthly mean minimum and maximum temperatures lie between 32 and 63° F., against 32 and 50° F. at Hurley. The summers are hot and dry. Hence these forms must grow at low temperatures or not at all. They appear to be better equipped to grow at British winter temperatures than native grasses. Against this, however, solar radiation is lower here (50–150 g. cal./cm.^{2}/day) in winter than in North Africa (250–400 g. cal./cm.^{2}/day) and the days are shorter (7½ hr. from sunrise to sunset at Hurley on 21 December against 9½ hr. in Algeria/Morocco).

MORPHOLOGICAL CHARACTERISTICS

Tiller production. The seasonal trends in tiller and leaf production, and in inflorescence initiation and ear emergence, were followed in spaced plants and in swards. The temperate forms produced new tillers vigorously from December to March, and again after flowering in July and August. The Mediterranean forms tillered in March, April and May, but were particularly vigorous from July to November. Flowering suppressed tiller formation in all forms.

Floral initiation and ear emergence. Floral initiation occurred in both temperate and North African forms in early February; S 170 and Alta (an American variety) were slightly the earlier. Ears of S 170 and Alta emerged in the first week of May and others about 15 days later. The interval from floral initiation to ear emergence was 90–95 days in all forms. About 60 % of all tillers were fertile (that is to say, they produced ears) in S 170 and Alta against only 22–32 % in the Mediterranean types. Thus reproductive behaviour is broadly similar in all forms, but the seasonal cycles of tiller production are very different.

Leaf production. The leaf area index was higher in the North African forms than in S 170 during winter, but lower in summer. The average size of eleven leaves (the first six and last five), out of 12·2 leaves formed between August and May on marked tillers of S 170 and a high-altitude Algerian form, showed that the first few leaves of S 170 formed in the late summer were bigger than those of high-altitude Algerian form, but all later leaves were smaller. The total leaf area of eleven leaves in S 170 was 150 cm.^{2} against 180 cm.^{2} in the high-altitude form.

From December to March, the North African types had more green leaves per tiller. A comparative score gave 2·9 in these forms against 1·8 in S 170. Thus, the North African types were greener than S 170 during winter.

The rate of leaf appearance per tiller was very similar in all forms. It was very slow during winter (1 in 54 days) compared with autumn and spring (1 in 20 days) and summer (1 in 10–15 days). There was a good correlation (r = 0·8) between the daily mean temperatures and the rate of production of new leaves per tiller.

Tiller elongation. There are important seasonal differences in tiller elongation between North African and temperate forms. Tillers were measured from ground level to the last visible ligule. Thus tiller elongation consists of expansion of the leaf-sheath under vegetative conditions, and during flowering it comprises elongation of both the leaf-sheath and the internodes of the stem axis.

In summer the elongation of the vegetative tillers was greater in S 170, giving it a more upright growth form than the North African types. During winter the vegetative tillers were more elongated in the Mediterranean forms and the plants also had a more erect habit than S 170. S 170 became somewhat decumbent during the very cold weather, and the decline in height shows that leaf sheaths may become shorter with the onset of very cold weather. During spring the internode elongation of the flowering tillers in the early type S 170 was more pronounced than in the rather later flowering Algerian high-altitude form.

PHYSIOLOGICAL CHARACTERISTICS

Changes in dry weight. During the period 1 November 1959 to 1 March 1960 spaced plants of North African types produced 23–27 g. of organic matter per plant, while S 170 produced the negligible amount of 6 g. per plant. It was shown by successive samplings that the increase in the North African plants represented true growth, i.e. there was a net increase in dry matter in all parts of the plants (leaf, stubble or leaf bases and roots). The slight increase in dry-matter production in S 170 was accompanied by a reduction of about 9 g. per plant in the content of soluble carbohydrates, particularly in the stubble, in which about 75 % of the total loss occurred. Root weight increased by 3–4 g. per plant in all ecotypes during winter. We see, then, that the North African forms not only produce more leaves during the winter, but also make far more true growth than the temperate forms.

Effects of temperatur and light. Since marked differences between the ecotypes in both morphological and ph· siological behaviour had been shown, it seemed important to analyse the reactions to temperature and to light. Plants of S 170 and the high-altitude Algerian form were

grown, in pots, from 24 October to 2 January, inside and outside a glasshouse, with and without supplementary light of 200 f.c. intensity given during the daylight hours by high-pressure mercury-vapour lamps. During November and December, S 170 was unable to respond in growth to either extra light or higher temperature unless the other were given; but the high-altitude Algerian form, whose growth was also greater than that of S 170 under normal outdoor conditions, responded to extra light outdoors and made rather better use of the higher temperature and of the supplementary light in the glasshouse. The stem weight (including leaf sheath) of S 170 decreased under all treatments except supplementary lighting inside the glasshouse. In both ecotypes the root weights decreased slightly when supplementary light was withheld.

Net assimilation rate. When the increases in dry weight during winter were further analysed it was found that, although both additional light and higher temperature increased net assimilation rate, the mean net assimilation rate of the high-altitude Algerian form was higher under all conditions (0·059 g./dm.^{2}/week) than that of S 170 (0·027 g./dm.^{2}/week). It is worth noting here that under summer conditions the values obtained for the net assimilation rates of the Moroccan form and S 170 were very similar. In the glasshouse from May to July, the respective figures were 0·49 and 0·40 g./dm.^{2}/week; and in June and July, in a field experiment harvested with a mower, the corresponding values were 0·14 and 0·13 g./dm.^{2}/week.

Leaf area. Analysis of the glasshouse experiment also showed that the high-altitude Algerian form had a larger green leaf area (15·7 dm.^{2}/pot) than S 170 (12·6 dm.^{2}/pot). Leaf area was unaffected by supplementary light but was greater in plants inside the glasshouse (15·7 dm.^{2}/pot) than outside (12·6 dm.^{2}/pot). The winter adaptation of the high-altitude Algerian form showed also in proportion of green leaf to total leaf by weight: in S 170 the value averaged 58 % but in the high-altitude Algerian form it was 71 %. Plants inside were greener (68 %) than those outdoors (60 %).

Effects of nitrogen. In the same experiment the high-altitude Algerian form was markedly more responsive to nitrogen, the mean response to an extra 120 p.p.m. of nitrogen in the nutrient solution being 3·0 g. per plant in S 170 and 8·5 g. per plant in the high-altitude Algerian form. Under winter conditions the higher level of nitrogen increased net assimilation rate, leaf area, and greenness in all cases and the response was particularly marked under low light and temperature conditions.

CONCLUSION

We have found then, that the winter growth of the North African tall fescues arises partly from their seasonal pattern of tiller and leaf production, but also from an inherently greater physiological efficiency under winter conditions. In addition to its greater leaf area, the Algerian form appears to have a higher net assimilation rate than S 170 at winter temperature irrespective of light intensity. A grass with this marked ability to grow at low temperature is clearly of great agronomic as well as physiological interest.

I gratefully acknowledge the guidance of Dr W. Davies, Prof. A. H. Bunting and Mr J. O. Green in this work.

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Reviews

Herb. I.M.I. Handbook. Commonwealth Mycological Institute, Kew. Pp. 103; 6 plates and 8 figs. 1960. 12*s.* 6*d.*

This most useful little book, which is dedicated to E. W. Mason, and was compiled by his colleagues for presentation to him on the occasion of his retirement, gives a brief history of the Institute (by S. P. Wiltshire), with information for reference on its present functions, publications and staff. For the rest it is devoted to accounts, in full working detail, of the methods in use at the Institute for the collection and description of fungi, the preparation of cultures, and the organization and maintenance of the Herbarium and culture collections. Typical of the helpful detail is the account (by H. A. Dade) of the use of nail varnish for the sealing of microscope slides—a simple expedient, found only after years of exasperation, that now saves a world of trouble. The handbook is *by* working mycologists *for* working mycologists: a friendly, indispensable, and almost intimate vade-mecum.

E. C. LARGE

Models and Analogues in Biology. Symposia of the Society for Experimental Biology No. XIV. Edited by J. W. L. Beament. Pp. vii + 255. Cambridge University Press. 1960. 50*s.*

'When I use a word', Humpty Dumpty said, in a rather scornful tone, 'It means just what I choose it to mean, neither more nor less.' Words to which he gave several meanings, and which were therefore worked particularly hard, were paid extra. Several speakers at this Symposium should have paid the word 'model' very highly indeed! They make it mean almost anything.

Many of the speakers, however, used the term model in what I believe to be a more or less legitimate way, to mean either a mechanical apparatus which reproduced some of the reactions of a living system, or a theoretical or mathematical scheme which might help to explain how a living system worked. These papers may be summarized in the words used in the article 'Models in Cybernetics', by F. H. George. 'This is clearly one way in which the rigours of mathematics might be brought to bear on the whole of biology.' Many find this approach valuable, illuminating and stimulating, and all biologists should be aware of it, even those whose minds cannot work in the same way.

On the dust cover we are told that this symposium 'discusses, in its broadest sense, the scientific method in biology. The contributions represent a wide range of scientific disciplines and the list of contributions reflects the diversity of their approach to this fundamental question.' Had the symposium been called 'Some views on the scientific method in biology' it would have given a clearer picture of the contents. However, the articles are mostly well written and of considerable general interest though I suspect that only the 'converted' will read them with understanding and those to whom they should prove most salutary will probably find them difficult to follow. The problem of communication between biologists is a difficult one, and I do not think this symposium does very much to solve it.

KENNETH MELLANBY

Introduction to a Submolecular Biology. By A. Szent-Györgyi. Pp. 135. New York and London: Academic Press. 1960. 36*s.*

The author believes that physiologists think too much in atomic or molecular terms. We need to approach the subject from a subatomic and submolecular viewpoint, to which we must add supramolecular qualities. It is an argument for looking at life as a series of stages in a complex system and for not concentrating exclusively on any one stage. He is here concerned mainly with what he regards as the first stage, the examination of cellular energetics in terms of molecular

and atomic electronics. The result is a brilliant and stimulating book containing not only a point of view not found in standard biochemistry texts, but also a wealth of information, and the references to it, some of which will be new to those whose formal study of chemistry stopped some years ago. The author pursues his course from photosynthesis, through cellular energetics, to consider briefly but provocatively such topics as the mechanism of drug action, the way ATP might drive muscle, and the functioning of the thymus gland, and finishes with the energetic basis of life itself. All physiologists and other biologists interested in the nature of vital processes should read this book.

D. W. WOOD

Phenolics in Plants in Health and Disease. Edited by J. B. PRIDHAM. Pp. ix + 131 and 18 pp. of plates. London: Pergamon Press. 1960. 42*s.*

This volume records the proceedings of the Plant Phenolics Group Symposium held at Bristol in April 1959. The great diversity of compounds with aromatic hydroxyl groups found in the plant kingdom is reflected in the papers presented. These are grouped into four sections: General, Lignification, Pathology and Genetics. The lignification section includes the chemistry and formation of lignin (F. A. Isherwood), the effect of light on lignin and leucoanthocyanin formation (T. Swain), and the importance of lignification in graft compatibility (G. Buchloh). The pathology section includes a wide range of topics: metabolism of aromatic compounds by fungi (D. Woodcock), the effects of phenolics on *Venturia* species (A. E. Flood), on *Gloeosporium perennans* (A. C. Hulme), of oxidized polyphenols on *Sclerotinia fructigena* (R. J. W. Byrde) and of tannins on virus infection (C. H. Cadman).

In the genetics section J. B. Harborne summarizes the variations in flower colour due to anthocyanins and co-pigments; R. C. Peckett surveys the phenolics in the genus *Lathyrus*. The danger of identification of a compound from an R_F value is stressed by E. C. Bate-Smith and J. B. Harborne in the discussion, and recently published work further emphasizes this pitfall. The general section includes an authoritative account of apple and pear tree phenolics (A. H. Williams), a review of the formation and possible function of phenolic glycosides (J. B. Pridham), work on betanin metabolism (S. P. Spragg) and on germination inhibitors of fungi and higher plants (C. F. Van Sumere).

The discussions and lists of references form a valuable part of the volume. The text is meticulously edited but the numerous plates are not all up to the same high standard. Many chemists and all botanists will find much of interest, but most of the information is now available elsewhere and this lavish production with its high cost is probably a luxury which most will have to forgo.

G. C. WHITING

Sorauer. Handbuch der Pflanzenkrankheiten. Volume 6, *Pflanzenschutz.* Part 3, second edition. *Biologische Schädlingsbekämpfung.* By Dr J. M. FRANZ. *Die technischen Mittel des Pflanzenschutzes.* By Dr ING H. KOCH and Dr H. GOOSSEN. Berlin. 1961. Paul Parey. DM 190.

Some 20 years ago when the sixth volume of *Sorauer* was first published, it was said that plant protection was a rapidly growing subject. Few people could then have imagined just how rapidly its development was to accelerate within a few years. The second edition is therefore not premature: Part 1 of it was published in 1952 and Parts 2 and 4 are in preparation; Part 3 marks the half-way stage in revision. Perhaps revision is not the right word, for the authors are mostly new (all new in the present case) and there is a great deal of new material re-arrangement and rewriting; Dr Franz's contribution, for example, bears very little resemblance to Dr Sachtleben's original. In an earlier review (*Annals*, **24**, 675) an outline was given of the subjects to be dealt with in Volume 6 and they are substantially unchanged.

The literature on biological control is extensive but scattered, and there are very few textbooks on the subject. Sweetman's books *The biological control of insects* (1936) and *Principles of biological control* (1958) are the most important, and to them must be added Sachtleben's (1941) contribution to *Sorauer* although it is not quite comparable. Dr Franz has now provided a most detailed, thorough and valuable addition to the list.

Biological control is one of the most fascinating fields of economic biology: its attractions are both practical and academic. In its classical context it has played only a small part in British plant protection, but in the wider sense outlined by Taylor (*Annals*, **42**, 190–6), it is of fundamental interest and it behoves the thoughtful plant protection worker to try to keep in touch with progress in the whole subject, especially in its broader aspects. Modern developments suggest that, even in the classical sense, the potentialities of biological agents should not be forgotten even in such a traditionally unlikely environment as our own.

Dr Franz's survey is concerned with the classical subject, and it is difficult to believe that he may have left out anything of importance. The contents of his work are shown in minute detail in a six-page table, and they are arranged most systematically. The introductory sections occupy 34 pages and, besides dealing with the history and theory of the subject, provide information on the national and international organizations concerned with it. Remaining chapters or sections (there are so many subdivisions, it is difficult to know what to call them!) deal with the use of micro-organisms, including viruses, protozoa, bacteria and fungi; arthropods (naturally by far the longest section), other invertebrates and vertebrates. The final chapters deal with the biological control of weeds and the problems and future tasks of biological control. This gives little indication of the detail: under the micro-organisms it ranges from diagnosis and identification through their role in natural population changes in insects, to the ways in which they can be used including their mass production and distribution. The main section on arthropods goes into equally broad but much greater detail.

The first edition of *Die technischen Mittel des Pflanzenschutzes* is the only previous text I have been able to trace covering virtually the whole range of apparatus used in plant protection and that was in pre-war years: the second edition (ready in 1957 which accounts for some omissions) is the only other one and it is almost completely new. Much of the content of the first edition is contracted into the historial section, and new sections deal, for instance, with safety equipment such as gas masks and guards on moving machinery and the official approval of spraying apparatus. The major part of the work deals with soil sterilization by physical and chemical methods, seed disinfection, spraying—including according to a descending scale of droplet size 'mists' and 'fogs'—and dusting. Other sections deal with such miscellaneous subjects as fumigation, pasteurization, irradiation, trapping and protection from frost. Under the various headings, the structural elements of machines and the types of apparatus available are described and illustrated and the economics of the different methods of applying chemicals to crops are discussed.

The book is well produced and copiously illustrated, though some of the charts and diagrams in the machinery section have suffered from reduction. The authors give the impression of having scanned world literature most thoroughly and their efforts have shown-up most strikingly the paucity of British publication on machinery and equipment.

In recent years, the editors of *Sorauer* have forsaken the use of extensive footnotes including all the references which now follow the main sections in fairly conventional style. The characteristic page is now much more attractive. The change of style started in the mid-1950's and has continued with minor modifications. Editorial policy is clearly active in this matter and on the whole has effected an improvement. The index is exhaustive and could not be faulted in a random check.

I have not set out to search for errors, nor is it worth while to list those I have noticed, but there are a number of typographical and other mistakes, particularly in the references. Generally the corrections are obvious. One curious error was noted, namely, 'Chemische Bodenuntersuchung' in the table of contents, p. xv, line 11, for 'Chemische Bodenunterseuchen' which appears on p. 321 of the text.

In dealing with a book in which the authors have clearly gone to great trouble and succeeded so well in producing a work worthy of wide use, I am reluctant to criticize, but cannot avoid the conviction that the publishers have produced an unhappy combination in this volume. That is in the sense that modern specialization is such that most people will be likely to want only one part, and that the high price of the volume (approximately £17) will prohibit them from buying it. One might congratulate the authors whose work is so highly valued but commiserate with them on having it priced out of the market.

F. H. JACOB

Ainsworth and Bisby's Dictionary of the Fungi. Fifth edition. By G. C. AINSWORTH. Pp. viii + 547. Kew: Commonwealth Mycological Institute. 1961. 30*s*.

The scope and pattern of this compact and scholarly work, which has reached its fifth edition in less than 20 years, is now very familiar to mycologists everywhere. The format remains the same though the title-page has sadly suffered a change because of the death of the co-author, Dr Bisby, whose portrait provides the frontispiece to this new edition. Much new material has been added, a fresh set of illustrations prepared, 500 more generic names have been compiled, and more than 5000 corrections or amendments have been made. The author can indeed truly claim that the content still reflects the *current* stage of mycological knowledge and practice. Suggestions to include the places of publication of all generic names listed and to indicate the types have been considered, but rejected for sound reasons, but as a compromise the dates of genera proposed between 1920 and 1939 have been included, so that the places of publication can readily be traced in Petrak's *Lists*, now reprinted as part of the *Index of Fungi* (C.M.I.) which covers new genera since 1940. Prof. Martin's Key to the Families of Fungi at the end of the book has also been carefully revised. Anyone who has used this dictionary regularly will appreciate that it is of the greatest value to the plant pathologist as well as to mycologists.

W. C. MOORE

Micro-Organisms as Allies. The Industrial Use of Fungi and Bacteria. By C. L. DUDDINGTON. Pp. 256. London: Faber and Faber, Ltd. 1961. 25*s*.

The thesis that, just as there are good fairies and bad fairies, there are also good bacteria as well as bad, has been a successful line for popular lecturers and writers for several years now. This book does the job well because although written primarily for the layman, it avoids the patronising air. Furthermore, it is of real value to a more sophisticated reader, a microbiologist who wants at any rate the principal details of those industrial biological processes with which he is not normally concerned. He should find here more detail than is usual in a book covering a fairly wide field. The details appear to be rarely inaccurate and where they are, they are of no grave importance.

A large proportion of the book is concerned with the activities of yeasts: beer, wine, spirits, industrial alcohol, baking and food yeast. Next come the lower fungi and actinomycetes, with obvious reference to the production of antibiotics and organic acids. The latest information on vinegar production is absent, but that may be because those engaged in that industry are still so coy.

The book contains a wealth of useful and interesting information without overdoing it, and the writer helps by putting over his story in a cheerful vein. The volume should be available in all teaching establishments and the cost is not excessive.

H. J. BUNKER

Principles of Plant Breeding. By R. W. ALLARD. Pp. xi + 485. London and New York: John Wiley and Sons. 1960. £3. 12*s*. 0*d*. ($9.00).

Starting with the dawn of plant improvement in Mesopotamia and ending with the role of induced mutations, this book is a welcome addition to the literature on the application of genetics to plant breeding. One wonders, however, whether much of the material is rather too advanced for undergraduate students of agriculture for whom the book is intended. A back ground knowledge of genetics is assumed, and indeed necessary, to make full use of the contents.

The main thesis is that there exists 'a basic unity of methods used in breeding self-pollinated species on the one hand, and cross-pollinated species on the other'. While possibly not as distinct in practice as in theory, this convenient division of the plant kingdom enables the author to present all the recognized methods of plant breeding in rational sequence. The general pattern of presentation is to consider first the genetic principles involved and then demonstrate the application of these to practice, with examples drawn from specific crops. It is refreshing to find that many of these examples are taken from recent work and are not mere repetition of standard references.

The remainder of the book consists of sections concerned with breeding for disease resistance,

polyploidy, interspecific hybridization, mutation breeding and the distribution and maintenance of improved varieties. This last section deals predominantly with the situation in America.

As the title implies, the book is almost entirely concerned with the genetic principles of plant breeding and their application. Details of techniques involved, biometry and experimental design are not included. On a very minor detail one may differ from the author in including strawberries with maize, walnuts and pecans as an example of monoecious crop plants even though monoecious strains may exist.

A highly selective but adequate bibliography is given at the end of each chapter and there is a useful eight-page glossary. The book is attractively produced, written in a pleasant and readable style and should find its way on to many bookshelves—if not those of agricultural students, at least those of their mentors.

D. WILSON

Fundamental Principles of Bacteriology (fifth edition). By A. J. Salle. Pp. 812. New York: McGraw-Hill. 1961. 85*s*. 6*d*.

This is a completely revised edition of Prof. Salle's well-known book with much new material added. 'A new chapter on Bacterial Genetics has been written by Dr W. R. Romig and incorporated in the text.' and Dr C. E. ZoBell has brought up to date the chapter on the 'Bacteriology of the Sea' which he prepared for the fourth edition.

The book has a strong chemical and medical bias and so is somewhat unbalanced, for if the treatment of these subjects is regarded as fundamental the same cannot be said of the meagre chapter on 'Bacterial and Viral Diseases of Plants' which consists chiefly of lists of names of the causal agents concerned and a brief discussion on the nature of viruses. One would have thought that at least a reference to the fundamental researches of Erwin F. Smith should have been included. In most of the other sections there are references to recent work both in the text and at the end of every chapter.

The book commences with a good account of the principle of the compound microscope to which is added a description of the electron microscope, though the brief paragraph on phase microscopy might well have been expanded. The chapter on 'The Morphology of Bacteria' is excellent, but it seems somewhat incongruous to follow on with brief chapters on Yeasts and Moulds in a work devoted to bacteriology.

There are some curious omissions as, for instance, in the paragraph dealing with pigments, so useful in identification and interesting in their chemistry; some are mentioned on pages 341–4 under 'chromogenesis' but fluorescin and pyocyanin are absent and the word pigment does not occur in the index.

It is to be regretted that the brief but concise account of the history and development of bacteriology which formed the final chapter of the second edition finds no place in this new one, for it is of fundamental importance to all engaged in the study of bacteria.

The chemistry of stains and the nutrition, respiration and enzymes of bacteria are all adequately dealt with as are also the subjects of sterilization and disinfectants. The illustrations, both as diagrams and as photographic reproductions, are excellent but the general tone of the work is medical and sanitary, very helpful for those engaged in searching for and identifying the colon bacillus in water and dairy products.

A most informative and somewhat expensive work by a recognized authority on subjects other than the bacterial diseases of plants.

W. J. DOWSON

Evolution: its Science and octrine. Symposium presented to the Royal Society of Canada in 1959. Edited by THOMAS W. M. CAMERON, F.R.S.C. Pp. xii+242. Published for the Society by University of Toronto Press. (Royal Society of Canada 'Studia Varia' Series 4.) London: Oxford University Press. 1960. £2. 0s. 0d.

The Implications of Evolution. By G. A. KERKUT. Pp. 174. (International series of monographs on pure and applied biology, volume 4.) Oxford: Pergamon Press. 1960. £1. 10s. 0d.

There was once a historian who maintained that history books were of two sorts: those that were good history but dull reading, and those that were good reading but bad history. 'The first', he said 'when I am reviewing, I call scholarly, the second brilliant.' The volume produced by the Royal Society of Canada to celebrate the centenary of *The Origin of Species*, is scholarly, both in the sense that one would expect any book written by learned men to be scholarly, and in the sense of our historian. Its twenty essays range from the details of the evolution of teeth to the origin of the elements, and from the processes of speciation to social evolution, but while many of them will be useful quarries of fact for the lecturer who has to teach off his speciality (here the scholarship is assumed) none can be recommended to a student for inspiration or enlightenment. This is partly because the catalogue type of approach adopted by some of the essayists does not easily lend itself either to generalization or to explanation, but more because the dominant attitude to evolution is that of the turn of the century. What, for example, will a student make of M. Y. Williams's statement about the Apterygota: 'These are primitively wingless, and are not definitely known before the Cretaceous but are probably ancestral to the subclass Pterygota known from Upper Carboniferous?' If he is dull, he will, like all dull students, either take no notice or memorize the sentence and serve it up in an examination. If he is either scholarly or brilliant (or perhaps if he has read Kerkut's book) he will be tempted to throw the symposium away and go no further. Or how will a student react when he finds B. F. Crocker basing his argument on, amongst other things, the exploded story of the dissimilarity of vertebrate and invertebrate phosphagens?

Biologists on this side of the Atlantic will take somewhat wryly G. M. Shrum's plea for a shift of emphasis in research from the biological sciences to the study of nuclear particles, and they will add that if his forecast: 'Canada will gain intangible but none the less valuable world recognition and stature from research in pure physics', is to come true, her scientists will have to do better than this book shows.

It would be easy to dismiss Kerkut's book as 'brilliant', and leave it at that. Some reviewers have come very near to doing so, adding only that he has not mentioned all the relevant facts. This is unfair since, as he makes clear in his first chapter, he is stating the evidence *against* what he calls 'the general theory of evolution' (in distinction from evolution in the sense of mutability of species) for the benefit of an undergraduate who has just presented him with a good essay on the evidences *for* evolution. These, then, may be assumed, and the book presents the arguments on the other side, to the effect that the unique origin of life and the genetic relationship of all living things are assumptions which are not biologically necessary. He covers a wide range and no doubt experts can find flaws here and there, but in total he presents a strong argument which should make students think. It must be read to be appreciated.

The sections on morphology might be improved by some generalization. Darwin believed that the gaps between groups could be filled by the discovery of missing links, and that is the simple deduction from, and indeed in the Baconian sense, test of, this hypothesis. In fact the reverse has happened; Darwin thought there were only four or five major groups; now the number is a dozen or a score, and it is increasing. The Coelenterata and Arthropoda have been divided since I was an undergraduate, probably the Protozoa will be ranked as two or three phyla within the next 20 years. At a lower level we have seen the fish split into three. Creatures such as *Branchiostoma* and *Peripatus*, so far from being missing links, turn out to be stumbling-blocks to our understanding of phylogeny. It therefore seems to be true that the more we know about the larger taxa, the more difficult it is to relate them to each other.

Perhaps Kerkut is being subtle or tactful, but his strictures on the undergraduate of today for swallowing opinions as facts, just like his theological predecessors, ought in justice to be

directed at the dons. Kerkut's book should be read and taken to heart, not only by undergraduates but by teachers and research workers (including Fellows of the Royal Society of Canada).

It is a pity, in view of its interest and importance, that the book was not better prepared; it reads in parts like a transcript of the off-the-cuff tutorial that it purports to be. For example, on p. 79, the word 'coelenterates' occurs six times in twelve lines, although the author cannot be unaware of the existence of pronouns, and on p. 137 the names of the vertebrate classes appear in mixed Latin and English forms, all with capitals. I hope that the book will have to be reprinted, and I hope that when this happens Dr Kerkut will give it a good polish. The publishers, too, now that they are established in Oxford, might usefully study the production and proof reading of their colleagues in Walton Street.

W. B. YAPP

Report of the Seventh Commonwealth Entomological Conference 6–15 *July* 1960. Pp. iii + 399. London: Commonwealth Institute of Entomology. 1960. 50*s*.

This volume of 400 pages falls into three main parts.

The first part (pp. 1–33) covers the formal business of the conference, the reports of the Directors of the Institutes of Entomology and Biological Control respectively and the results and recommendations of the Committees set up to consider them.

The second part (pp. 34–195) consists of thirty-one papers delivered at the fourteen open sessions and the discussions on the papers. The major subjects covered were insecticides and their uses (four papers), stored products infestation (three), relations between plant and insect (two), timber pests and termites (four), trypanosomiasis and tse-tse flies (two), locusts and insect dispersal (four), plant viruses (four) and the application of research (five).

The third part (pp. 196–399) is made up of reviews of work in economic entomology in Commonwealth countries since the previous conference in 1954. There are forty-seven reviews from thirty countries, including reports from regional research centres and specialist subject institutes.

In reviewing such a volume one may well first ask what are the objects of a Commonwealth Entomological Conference? Primarily they must be to give Commonwealth Entomologists the opportunity to review the work of the Institutes and to advise on their future development; secondarily to give them the opportunity to meet and discuss problems of mutual interest both formally and informally. Looked at in this light one criticism is that some of the sessions were overweighted both in principal speakers and in discussions, with matters mainly of interest to the United Kingdom, especially those of administrative procedure and organization. U.K. institutions accounted for 72 % of the papers in 1960, compared with 47 % in 1954.

It is to be hoped that at the next conference there will be fewer papers on more general topics with adequate time for discussion. Papers of this kind at the present conference were those of Wilson on biological control (p. 72), Harris on termites (p. 103) and Smith on insect pathogens (p. 111).

With the present tendency to use indiscriminate application of chemicals, it is refreshing to note the emphasis placed by many speakers—notably Kearns (p. 44), Hadaway (pp. 45, 48), Ford (pp. 125–6) and Rainey (pp. 152–6) on the need for thorough study of the biology, behaviour and ecology of insects as the basis for economical and effective use of chemicals.

As the conference itself resolved (recommendation 8, p. 9), the reviews of work in progress to the various territories would be more useful if they were shorter and more standardized. But this would be difficult to achieve and the Institute is to be congratulated on presenting in one volume a series of valuable scientific papers and a conspectus of entomology in the Commonwealth.

J. A. FREEMAN

Diseases of Vegetables. By L. OGILVIE. *Ministry of Agriculture, Fisheries and Food, Bulletin*, no. 123, fifth edition, 101 pages, 12 plates. London: H.M.S.O. 1961, 7*s.* 6*d.*

This is the fifth edition of a bulletin which is already well known to plant pathologists and others who are concerned with the growing of healthy vegetable crops. A great deal of information is condensed within the bulletin and the descriptions of individual diseases are necessarily brief. They are quite adequate, however, for a preliminary identification to be made and the advice on control measures is concise and practical.

The fourth edition was published in 1954 and since that time a number of vegetable diseases have assumed greater prominence or have been the subject of further research. Most of the recent information of such diseases is included in the present edition of the bulletin. Of particular note is *Fusarium* wilt of peas about which little was known in 1954 but which now causes much concern in many pea-growing areas. It is described in the present bulletin together with notes on resistant varieties. Other diseases on which more recent information is now included are watercress crook root, beet silvering, pea viruses, tomato leaf mould and tomato stem-rot. The sections on fungicides and seed treatment have also been considerably expanded.

The type and general layout of the bulletin represents a great improvement over the 4th edition although, in the opinion of the reviewer, the individual crops would be easier to find if they were arranged in alphabetical order. A few of the photographs are not quite as good as could have been desired. These relatively minor criticisms are well offset, however, by the overall excellence of the publication which is a reference work of very considerable value.

W. G. KEYWORTH

Biochemistry of Insects. By DARCY GILMOUR. Pp. 343. New York and London: Academic Press. 1961. 64*s.*

Insects possess a nervously co-ordinated physiology remarkably analogous to that of higher animals. They have, therefore, provided very convenient material for biochemical investigations of nervous and muscular function. The spectacular phenomena of insect metamorphosis, flight, pigment, and silk production have similarly provided a fascinating challenge to the comparative biochemist and endocrinologist.

The successful control of insect vectors of disease and pests of agriculture by chemicals and the profound biological implications of insect resistance have emphasized an urgent need to study the biochemistry of insecticidal action and inaction. For these reasons, there has been a mushroom-like growth in the body of knowledge of the biochemistry of insects and this has become unusually widely scattered in the literature.

Dr Gilmour is to be congratulated on being the first to attempt its integration within a single tome. Any book on such a rapidly expanding subject can never be quite up to date, and this is illustrated by the book under review in which (on page 200) it is stated that no digestive chitinase is known in insects. Within the last few months, a digestive chitinase has, in fact, been demonstrated in at least one species by Waterhouse and his colleagues.

Despite its title, the book tends, perhaps, to be a conventional introduction to biochemistry with particular reference to insects, and, on this account, may disappoint the specialist. For example, more attention to the beautiful work of Bücher's school on the comparative biochemistry of locust muscle would have been more rewarding than the well-known descriptions of carbohydrate catabolism in vertebrate tissues. Despite these minor shortcomings, the book is unique and well written. Very few inaccuracies were found and none was serious. For example, Baldwin and Needham found arginine phosphate in blow-flies, not in house-flies (page 144). The author has very usefully emphasized some of the important gaps in our knowledge and has not hesitated to mention weaknesses of existing evidence.

The book is well bound and clearly printed. It is strongly recommended for the use of teachers and students of comparative biochemistry and entomology. F. P. W. WINTERINGHAM

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